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Peptides Quantification by Liquid Chromatography with Matrix-Assisted Laser Desorption/Ionization and Selected Reaction Monitoring Detection

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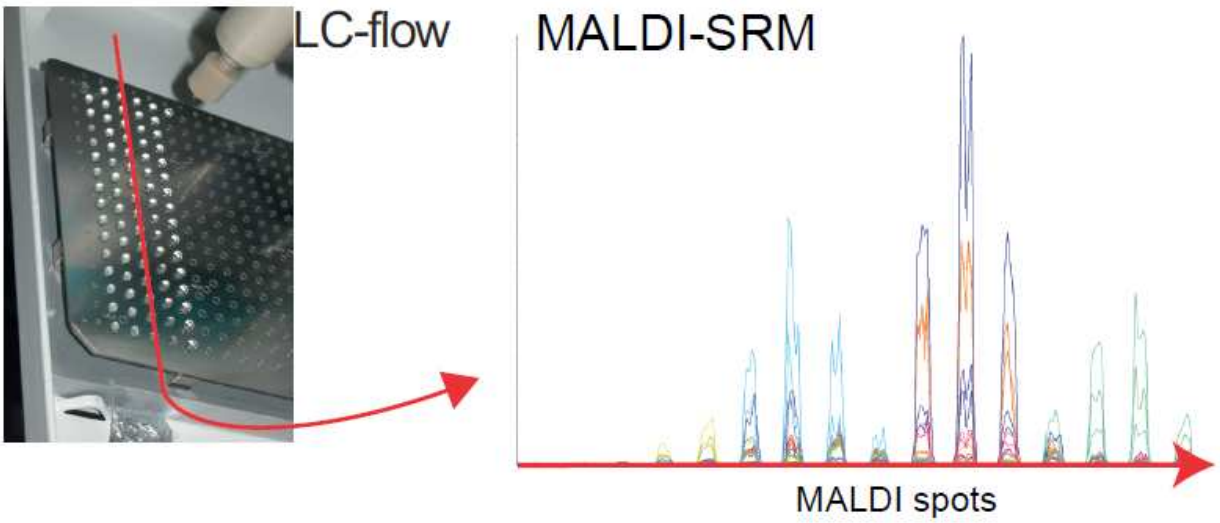
Abstract

We present a novel analytical platform for peptides quantitative assays in biological matrices based on microscale liquid chromatography fractionation and matrix-assisted laser desorption/ionization mass spectrometric detection using the selected reaction monitoring (SRM) mode. The MALDI source was equipped with a high frequency Nd:YAG laser (1000 Hz) and mounted on a triple quadrupole / linear ion trap mass spectrometer (MALDI-QqQ_{LIT}). Compared to conventional LC-ESI-SRM/MS, the separated analytes are "time-frozen" onto the MALDI plate in fractions and navigation through the LC chromatogram makes possible to perform SRM experiments as well as enhanced product ion spectra acquisition for confirmatory analyses without time constraints. The LC spots were analyzed using different rastering speeds ranging from 0.25 to 4 mm/sec with the shortest analysis time of 425 ms/spot. Since the LC runs can be multiplexed and do not need a comprehensive investigation, the present platform offers a valuable alternative to LC-ESI-SRM/MS for high throughput proteomic analyses. In addition, the derivatization of the N-terminal α-amino group by sulfonation was found to be key for the fragmentation of singly charged peptides under low collision energy regime. Under such conditions, y-ion series were observed in the MS/MS spectra, and thus the design of SRM experiments was greatly simplified. The quantitative performance of the platform was compared to that of LC-ESI-SRM/MS by spiking yeast tryptic peptides in human plasma digests. Both platforms exhibited similar sensitivities, accuracy (within +/-20%) and precision (under 20%) in the relative quantification mode. As a proof of principle, the relative and absolute quantification of proteins associated with glycolysis, glyoxylate and tricarboxylic acid (TCA) cycles over a growth time course of *Saccharomyces cerevisiae* on glucose media, was successfully performed using isotopic dilution.



Keywords: Peptides, MALDI, quantification, SRM, yeast, chemically assisted fragmentation.

Introduction

The targeted mass spectrometry (MS) approach using a triple quadrupole operating in selected reaction monitoring (SRM) mode for the detection and quantification of peptides (as surrogates of their proteins) has gained in importance during the last decade. Experiments based on selected reaction monitoring, by contrast to the unsupervised shotgun approach requires the preselection of peptides representing the proteins of interest. Such peptides have to fulfill some particular criteria, including uniqueness of the amino acids sequences, MS-observable as tryptic products, and exhibiting collision-induced fragment ions) prior to perform the LC-SRM/MS analysis [1]. The SRM quantification, well established for small molecules analyses, has been recently applied for the absolute quantification of biopharmaceuticals and biomarkers [2-4] as well as for monitoring large sets of protein [5-10]. The high selectivity of the SRM acquisition reduces significantly the need of sample fractionation and considerably simplifies post-acquisition data processing. Picotti *et al.* have demonstrated that a 30 minutes LC gradient is sufficient for the monitoring of the glycolysis, tricarboxylic acid (TCA) and glyoxylate cycles proteins network of *S. cerevisiae* with sensitivity down to 50 proteins/cell and additional detection of previously undetected proteins [11].

Nevertheless, LC-MS using electrospray ionization has limited analytical throughput and acquisition flexibility due to the intrinsic hyphenation between the chromatography and the mass spectrometer. On the contrary, matrix-assisted laser desorption ionization (MALDI) allows high throughput capabilities because the LC separation step is decoupled from the MS measurements. This promotes multiplexing the LC separation by fractionating simultaneously 2-4 samples onto the same MALDI plate, and a subsequent in-depth MS analysis. Currently most LC-MALDI assays are run on TOF/TOF instrumentation using high energy collision induced dissociation (CID) [12]. MALDI ionization produces almost exclusively singly charged protonated peptides that do not usually provide reliable MS/MS spectra under low energy CID. Therefore, high energy CID is generally required to fragment singly charged peptides and the resulting MS/MS spectra can be complex exhibiting a, b, c, x, y, z series, internal and side-chain cleavage fragments d, v, w [13], which may affect the overall sensitivity of the assay. A chemically assisted fragmentation step is frequently applied to support TOF/TOF fragmentation of peptides and simplify the spectra interpretation with an enhancement of the signal to noise ratio [14-16]. The chemically assisted fragmentation [16-19] consists in derivatizing the tryptic peptides using sulfonation reagents such as 4-sulfophenylisothiocyanate (SPITC). It is postulated that the strong negative charge, *i.e.* given by the sulfonate moiety localized at the N-terminus of the peptides, forces them to carry two protons in order to be ionized in positive mode [17]. The sulfonated peptides remain singly charged but fragment readily due to the presence of two protons.

The combination of a triple quadrupole with a MALDI source is a recent setup [20-22], which has been applied mainly in quantitative analysis of pharmaceuticals [23, 24], and MALDI-MS imaging of low molecular weight compounds [25, 26]. This particular configuration encompasses a high frequency laser (up to 1000 Hz) in order to produce a quasi-continuous ions beam that is mandatory for SRM experiments. During the acquisition, the laser is operated in rastering mode and passes through each MALDI spot consuming only a small percentage of the total spot surface per run. This feature offers a convenient re-analysis capability and flexibility, which allows defining and optimizing the SRM transitions after the LC fractionation and MALDI deposition steps. Alternatively direct quantification of low molecular weight compounds using high resolution MALDI-TOF [27] has been described and recently extended to

peptides quantification including an immunocapture step of the peptides in order to maintain the selectivity [28].

In the present work, we have investigated the potential of the LC-MALDI-SRM/MS platform to analyze complex biological samples. The specific optimization of the analytical platform is discussed and illustrated by monitoring a set of proteins from the TCA and glyoxylate cycles, and of the glycolysis and gluconeogenesis pathways in *Saccharomyces cerevisiae*.

Experimental section

Yeast culture and proteins extraction

The time course of *S. cerevisiae* cells strain SC288 (ATCC 26108) was performed on 400 ml of liquid YPD broth (Sigma Aldrich) in a 1L Erlenmeyer flask at 28°C. The agitation was performed using a magnetic stirrer. The culture was inoculated with 5 mL of a 24 H pre-culture (OD=8) grown in liquid YPD medium from a single colony harvested on a Sabouraud agar dish. The commercial YPD broth consist in bacteriological peptone 20 g/l, yeast extract 10 g/l, glucose 20 g/l. The cells concentration in growth medium was measured with a hemocytometer throughout the time course as well as the optical density (OD) at 600 nm. Cells were harvested at 10 time points: 9, 10.5, 12, 20, 23, 25, 27.5, 30, 47 and 49.5 hours. At each time point, a volume of the culture was immediately cooled in ice-cold water and then pelleted by centrifugation (1000xg, 5 min, 2°C). Pellets were washed with an ice-cold buffer (HEPES 20 mM, EDTA 2mM, pH 7.5), subsequently frozen in liquid nitrogen and stored at -80°C. The glucose concentration in growth medium was determined by a hexokinase enzyme assay (Aldrich) on the supernatants resulting from the harvest.

The pellets were solubilized in a lysis buffer (HEPES 50 mM, glycerol 5%, dithiothreitol 15 mM, KCl 100 mM, EDTA 5mM, pH 7.5 and a complete protease inhibitor cocktail (Roche), one tablet for 25 ml of lysis buffer). Cells numeration using a hemocytometer was performed in triplicate on the aliquots of the solubilized pellets. This numeration was used to rationalize the proteins expression profiles expressed in proteins/cell. Cells disruption was performed using 500 µm acid-washed glass beads. Proteins concentration of each extract was determined using a BCA assay (Pierce). Proteins extracts were precipitated with 8 volumes of cold acetone overnight at -20°C and stored at -80°C until further use.

Tryptic digestion and peptides sulfonation

The proteins pellets were solubilized in 320 µl of 8M urea and 0.1M ammonium bicarbonate buffer, reduced with 20 µL of 0.5M dithiothreitol at 30°C for 45 minutes and then alkylated with 60 µl of 0.5 M iodoacetamide for 1 hr at room temperature. Aliquots of 300 µg of proteins from the subsequent solutions were diluted with 0.1M ammonium bicarbonate in order to reduce urea concentration below 0.5M (final volume 1470 µl). The isotopically labeled peptides standard mixture (C terminal arginine, $^{13}C_6$, $^{15}N_4$, Δm=10 u, JPT Peptides Technologies), used for precise quantification, was spiked in each sample (30 µl, 50 pmol) before trypsin addition (trypsin/total proteins ratio of 1/75; w/w, total protein amount of 300 µg).

Digestion was performed overnight at 34°C under agitation; the trypsin activity was quenched by neat formic acid addition (ca. 20 µl). Samples were desalted onto C18 solid phase extraction cartridges and subsequently evaporated to dryness in a vacuum centrifuge. The digest pellets were solubilized with a

small amount of MeCN/TFA 0.1% (20/80; v/v) vortex-mixed and sonicated before being diluted with aqueous 0.1% TFA solution, aliquoted and stored at -80°C until further analysis.

Peptides from each time point were derivatized in triplicate by the following procedure. An equivalent of 50 µg of the digested sample was evaporated to dryness and subsequently solubilized with 50 µL of sulfonation reagent (sodium bicarbonate 20 mM, 4-sulfophenylisothiocyanate 20µg/µl, pH 10). The sulfonation reaction was performed at 55°C for 45 min. under agitation. The 4-sulfophenylisothiocyanate and other reaction by-products were removed by steric exclusion (cut-off of 700 Da) using a homemade "SpinTrap"-like device packed with Sephadex G10. Elution and equilibration steps were done with a 20/80 (v/v) ethanol/water solution. The filtrate was evaporated to dryness, solubilized in 10 µl of 1% aqueous TFA solution and further diluted (7 fold) in 0.1% TFA to obtain a final pH below 2.

Microscale LC-MALDI

The microscale LC-MALDI fraction collection consists in a column-switching setup with a trapping column (1 mm i.d. × 5 mm, 2.7 µm Halo C18 fused-core, Optimize Technologies) and an analytical column (1 mm i.d. × 150 mm) packed with the same stationary phase. The loading mobile phase consists of 0.1% aqueous TFA delivered at a flow rate of 200 µl/min. A low-pressure binary linear gradient from 5 to 60 % B in 15 minutes was delivered through the analytical column by an UHPLC pump (Rheos Allegro, Flux Instruments). The eluent A consists of water/MeCN/formic acid (95/5/0.1; v/v/v) and eluent B in MeCN/water/formic acid (95/5/0.1; v/v/v), the total analytical flow was set to 50 µl/min. Samples (35 µg/injection) were loaded for 1 min. onto the trapping column before being eluted onto the analytical column.

A solution of α-cyano-4-hydroxycinnamic acid (CHCA; 20 µg/µl in 90/10 MeCN/0.1% aqueous TFA) was infused at 10 µl/min and mixed to the mobile phase flow stream in the MALDI spotter (PAL-HTS autosampler equipped with MALDI Spotter option, CTC Analytics). Fraction collection was carried out onto hydrophobically coated MALDI plates (µFocus-1700µm, Hudson Surface Technology) at a rate of 4s/spot. After drying, the MALDI spots were recrystallized by adding 1 µl of MeCN/0.1 %TFA (70/30; v/v) in order to improve crystals adhesion to the MALDI plate.

Evaluation of the quantification performances

Yeast's phosphoglycerate kinase (PGK) was purchased from Sigma Aldrich. Dilutions and sample preparation were performed in order to generate the following amounts: 25 ng, 50 ng, 100 ng and 1000 ng in terms of intact protein equivalent injected onto the column. The four phosphoglycerate kinase samples were spiked with a constant amount of peptides internal standard (C terminal arginine, $^{13}C_6$, $^{15}N_4$, Δm=10 u, JPT Peptides Technologies) and digested in triplicate by trypsin. The PGK digests were spiked into human "equalized" plasma that was previously digested by trypsin. The "equalized human plasma" was obtained by a partial proteins precipitation with MeCN before the digestion procedure in order to "equalize" the proteins concentrations range. In addition, the total proteins concentration was determined using a BCA assay (Pierce) in order to generate a well-characterized amount of matrix background. In our case 13,4 µg of digested plasmatic proteins were injected onto column along with the phosphoglycerate kinase. The peptides sequences of the yeast protein were blasted (NCBI Blastp

algorithm) against the human proteome to confirm their uniqueness. For the comparison of the quantification performances between the LC-ESI-SRM and the LC-MALDI-SRM platforms the same LC setup (LC system, column) was used. For the LC-MALDI experiments, samples were derivatized by SPITC, purified by steric exclusion and finally injected onto the chromatographic system. For the LC-ESI experiments, samples were kept native and the analysis was performed on a 4000 QTRAP equipped with a TurboIon electrospray source. The SRM transitions of the native peptides were selected from SRMAtlas (http://www.srmatlas.org/). The same amount of sample was injected on both platforms.

MALDI SRM/MS analyses and data processing

MALDI-SRM/MS analyses were performed on a 4000 QTRAP mass spectrometer (QqQ_{LIT}) equipped with a prototype MALDI source (AB Sciex). The Nd:YAG laser (355 nm) can reach the frequency of 1000 Hz in order to produce a quasi-continuous ions beam. The 4000 QTRAP mass spectrometer was operated in SRM mode with the Q1 and Q3 analyzers set at unit mass resolution. The SRM transitions of the selected peptides were acquired with a dwell time of 10 ms. In each case, transitions corresponding to the endogenous peptides and the internal standards were measured. During the acquisition the laser passes through each spot in rastering mode, and for each MALDI plate column a specific SRM transitions set, dedicated to peptides known to be collected along the column, was monitored. Three SRM transitions were monitored for each surrogate peptide. The SRM traces integration was manually done with MultiQuant software (v. 2.1, AB Sciex) using the summation algorithm, the ratio between the light and heavy transitions was calculated. The limit of detection was set to a S/N of 3, where the same number of preceding adjacent spots, typically 1-4, was taken for calculating the noise as for determining the analyte summed areas. The internal standard interference (cross-talk) was assessed by injecting the heavy peptides alone and by monitoring the SRM transitions corresponding to the light and heavy peptide pairs and not found to be critical in the present study. As the for low molecular weight compounds and this study is a proof of concept, only the most sensitive SRM transition was taken, the others being used as confirmatory transitions. In addition, targeted peptide identities were confirmed by a full scan MS/MS spectrum in linear ion trap mode on a representative LC-MALDI run of the yeast digest. For the quantification of the proteins, only the peptide showing the highest concentration result with the absolute quantification was taken into consideration when several peptides are available. Precise quantification (equation 1) was calculated using the ratio between the light and heavy peptide SRM peak area multiplied by the known amount of heavy peptide and corrected by the number of cells extracted per volume of injection. Results were expressed in number of molecules per cell. Relative quantification (equation 2) or "fold change" was obtained from the ratio between the light and heavy peptide SRM peak normalized to the reference growth time point.

$$concentration\ (^{molecules}/_{cell}) = \frac{\frac{light}{heavy} SRM\ ratio \times moles\ of internal\ standard\ injected \times N_A}{number\ of\ cells\ extracted\ per\ injection\ volume} \quad \text{Equation 1}$$

$$fold\ change = \frac{\frac{light}{heavy} SRM\ ratio \times k}{\frac{light}{heavy} SRM\ ratio\ at\ reference\ growth\ time\ point} \quad \text{Equation 2}$$

with k = correction factor based on the number of cells extracted per injection volume.

Results and discussion

Analytical characteristics of the MALDI-SRM/MS platform

Peptides fragmentation on a MALDI-triple quadrupole MS platform

An important analytical challenge of a LC-MALDI-SRM/MS platform for the quantitative analysis of peptides was the lack of an efficient and reliable/predictable fragmentation of singly charged native peptides, using the low energy collision cell. This limitation is intrinsic to the MS type used in the actual analytical platform, which contrasts to the high energy fragmentation of peptides in the commonly used MALDI-TOF/TOF instrumentation[29, 30]. However, a peptide derivatization step prior to their separation can overcome this limitation, resulting in predictable and reproducible fragmentation pattern of the peptides, which is mandatory for SRM experiments.

The use of SPITC as chemical derivatization agent enhances and simplifies dramatically the fragmentation of singly charged peptides and has been described for qualitative analysis[18, 31,]. Wang and coworkers [32] further optimized the prodedure and reported high conversion rate (>90%) for a model peptide and a tryptic digest. In the present study using the same derivatization procedure it was observed that the conversation rate was peptide dependent and superior to 50 % as illustrated in figure S5 (supplementary data). In general, for most of peptides investigated the conversion rate was found to be analyte dependent and reached a maximum within the first hour. More important than the conversation rate is the reproduducibility of the derivatization step for quantitative analysis. Previous work[29] and the present study show that quantification is not affected by the derivatization step as the labeled peptide is added to the sample and undergo the same reaction. Due to the lack of characterized reference the absolute response factor could not be determined for the derivatized peptides, but it was measured that the derivatization step under MALDI ionization did not result in a significant change of the signal in full scan MALDI mass spectra, suggesting that the ionization efficiency remains similar for both forms of the peptides. For quantitative analysis of sulfonated peptides further improvements are required: i) the determination of the optimal collision energy for a specific precursor ion mass, and ii) the removal of the hydrophobic derivatizing reagent excess by steric exclusion prior the LC chromatography. By monitoring 261 SRM transitions corresponding to 37 derivatized peptides, a linear regression equation between the collision energy and the peptide *m/z* was obtained: CE (eV) = 17.5 + 0.044 × *m/z* (r^2 = 0.92) (see figure S2 of supplementary data). This equation was applied throughout the study to optimize the fragmentation of SPITC-derivatized peptides. In addition, sample preparation was improved by adding a gel filtration step to remove the SPITC excess, which impedes MALDI matrix crystallization and contaminates the LC separation. Further discussion and data are available as Supplementary Material.

Matching the MALDI plate speed to the SRM/MS duty cycle

The source of the MALDI-QqQ_{LIT} mass spectrometer combines a fast moving target plate (from 0.1 to 30 mm/s) and a high repetition rate laser (up to 1000 Hz). This particular setup modifies the current acquisition concept inherited from ESI-SRM/MS, thus allowing more flexibility during the acquisition.

With SRM detection the maximum number of transitions monitored during a single run depends of i) the MS duty cycle (*e.g.*, for the 4000 QTRAP the fastest duty cycle per SRM transition is of 10ms corresponding to 5ms for the transition dwell time and a 5 ms pause time between each transition), and of ii) the analyte's availability for the MS detection depending on the hyphenated technique timescale (*i.e.*, few seconds for a chromatographic LC peak to several minutes for an infusion experiment). When using LC-ESI-SRM/MS, the number of peptides transitions monitored per run is limited by the chromatography taking into account that at least 10-15 data points across the peak were required for an adequate definition of the elution profile and thus, integration precision. However, it is possible to monitor hundreds of SRM transitions during a LC run by using a scheduled SRM acquisition, but this implies that the analytes retention times should be determined in a preliminary analysis [33].

Alternatively, peptides MALDI-SRM/MS analysis can be performed until the whole MALDI spot surface has been rastered and the sample consumed. Moreover, the integration of the MALDI-SRM/MS signal requires less data points because a rectangular pulse shape is generally observed and does not need a peak definition algorithm. In addition, the MALDI plate speed can be adapted to match the MS duty cycle in order to acquire enough points across the MALDI spots. As an example Figure 1 shows that the acquisition time needed for monitoring a single peptide (*i.e.*, 6 SRM transitions for the heavy/light peptides pair) can be as short as 425 ms per spot. When increasing the number of SRM transitions to be monitored (*e.g.*, up to 72 transitions), lowering the plate speed (*i.e.*, from 4 to 0.25 mm/s) will balance the increased MS duty cycle in order to keep the MALDI spots sampling rate adequate.

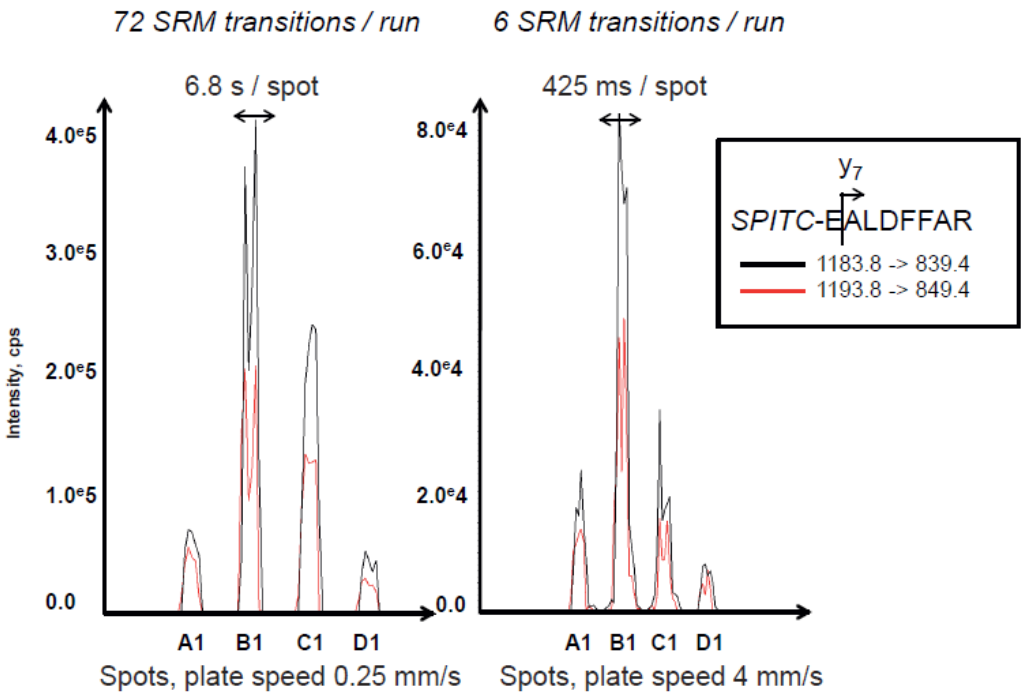


Figure 1: *SRM traces of the light and heavy y_7 fragment ions of the sulfonated peptide EALDFFAR. The SRM traces are extracted from a 72 (left) or 6 (right) SRM transitions set acquired with a MALDI plate speed of 0.25 mm/s and 4 mm/s respectively.*

The hydrophobically coated MALDI plates with the largest spots diameter of 1.7 mm were selected to concentrate droplets of maximal volume; however, droplets larger than 4 µl started to spill over the spot edges preventing the hydrophobic coating to concentrate the analytes during the evaporation step. Therefore, the matrix and LC flowrates had to be adjusted accordingly, as well as the fraction collection rate. With the current microscale LC setup, peptides were eluting over four fractions as shown in Figure 1. Ideally, a capillary LC setup with a flowrate of 4-5 µl/min and a similar separation power would be beneficial for the peptides elution in a single spot, but the use of the summation algorithm for integrating the four spots altogether did circumvent this limitation. Further discussion about LC flow-rate and sampling frequency is given in the Supplementary Material.

Spatially-mapped MALDI-SRM/MS acquisition strategy

As shown in Figure 1, the area ratios of the light/heavy peptides are not dependent of the plate speed. Therefore, it is possible to generate a "spatially-mapped" SRM acquisition (*i.e.* similar to the LC scheduled SRM strategy) with the adaptation of the MALDI plate speed according to the number of SRM transitions that have to be monitored in a MALDI spot for an optimal throughput. This approach is similar to the LC peak parking technique used with LC-ESI-MS [34], but with the advantage of the sample

re-analysis capability inherent to the MALDI process. Indeed, one could devise a data-dependent acquisition experiment monitoring the peptidic spots content during the MALDI plate rastering. The survey scan would trigger SRM experiments and the plate speed would be adapted according to the number of SRM transitions to monitor in order to get enough data points across each spot. Compared to LC-ESI-SRM/MS the chromatographic separation is "time-frozen" onto the MALDI plate during the fraction collection step and navigation throughout the LC run becomes possible without time constraint to devise SRM transitions "on-the-fly". After a rapid SRM screen to determine on which spots the peptides of interest are located on the MALDI plate, optimized SRM methods are created and dedicated to the peptides present in each spot.

Workflow to select the SRM transitions

Selection of the SRM transitions for peptide analysis with an ESI-SRM/MS platform

Typical LC-ESI-SRM/MS-based proteomic assays monitor surrogate peptides (so-called signature peptides) for protein quantification. For each signature peptide this requires certain knowledge of the precursor ion charge state, its fragment ions *m/z*, as well as the selectivity and sensitivity of the chosen SRM transitions. The growth of validated SRM assays databases (*e.g.*, SRMAtlas [35]) will probably simplifies this time-intensive process. When the targeted peptides are not present in those databases, the adequate strategy for building an SRM-based assay depends on the actual number of proteins to monitor. For a small set of proteins (*i.e.*, 10-20 proteins), the selection of the adequate signature peptides and the CID fragmentation optimization can be performed directly on the digest of purified proteins. However, the commercial availability of such protein standards is not guaranteed and this approach can be expensive for a large-scale proteomics screening. Therefore for larger sets of proteins, an alternative strategy consists of extracting the peptides sequences and information about their presence in digested biological samples from databases of peptides experimentally observed by mass spectrometry (*e.g.*, PeptideAtlas [36]) or from in-house unsupervised MS(/MS) experiments [37]. Then the SRM transitions can be easily predicted by an *in silico* fragmentation software and further optimized with synthetic peptides [11] or directly extracted from full scan MS/MS spectra. However, these approaches require that a preliminary screening of the biological samples should be undertaken with an initial SRM transitions set in order to confirm the targeted peptides presence and, if necessary, to further optimize the SRM transitions. As a matter of fact, all these strategies to design the optimal SRM transitions set are time-consuming mainly because of the inherent speed and serial practice of liquid chromatography itself. Furthermore the maximum number of SRM transitions monitored per run remains limited and requires reliable information about peptides elution times to apply scheduled SRM approaches.

Selection of the SRM transitions for sulfonated peptides with a MALDI-SRM/MS platform

The flexibility gained by the re-analysis capability of the MALDI technique is of upmost importance for the development of quantitative MALDI-SRM-based proteomic assays. For small proteins sets MALDI can easily analyze the digest of a single protein without the need of an LC fractionation. The complete method optimization, *i.e.*, from the surrogate peptides selection to their CID fragmentation optimization and SRM transitions design can be quickly performed with a single MALDI spot (Figure 2a).

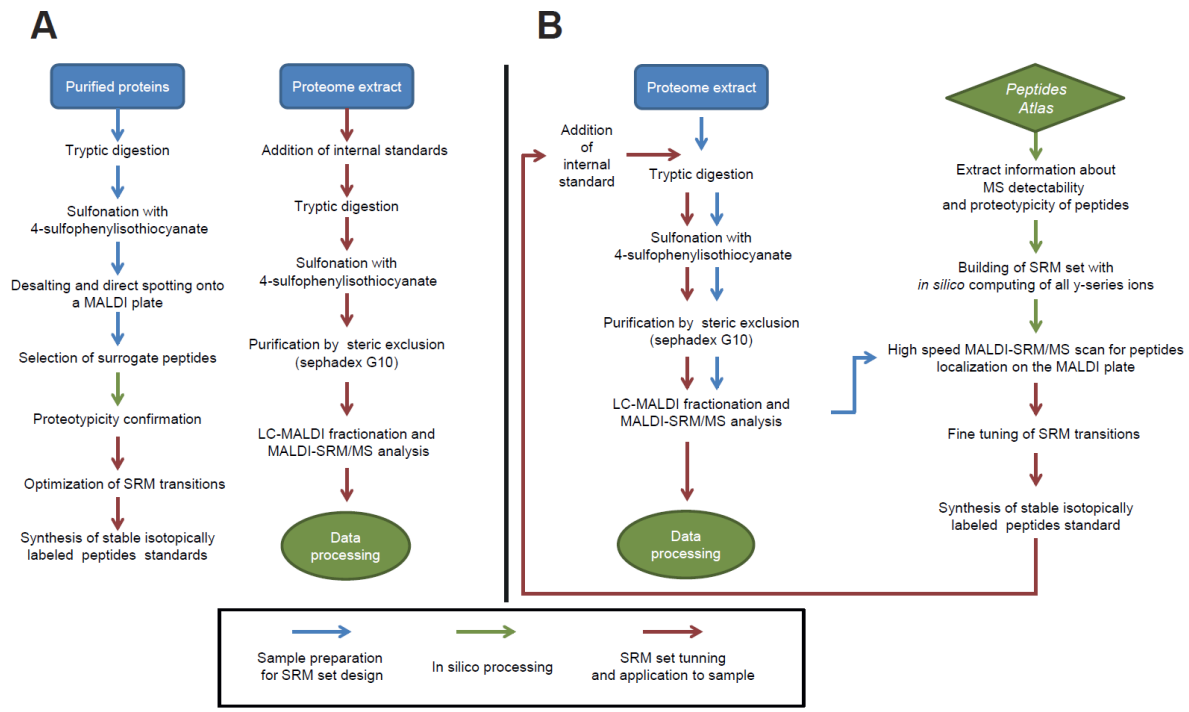


Figure 2: *Workflows for the SRM transitions set design when analyzing a) a small set of proteins or b) a large set of proteins.*

The second workflow to design large-scale SRM experiments using the LC-MALDI-SRM/MS platform is described in Figure 2b. Since the liquid chromatography separation and the MS detection are decoupled, the SRM transitions sets can be directly optimized on a single representative biological sample after LC-MALDI fraction collection in order to preserve the study samples. Despite the fact that SRM assays databases for SPITC-derivatized peptides are not currently available, the information about peptides detectability in similar biological samples can be extracted from databases such as PeptideAtlas. Despite of the fact that PeptidesAtlas is composed mainly of ESI-MS data, in many cases (provided the constraint of having arginine as C-terminal amino acid residue), the peptides selected in this MALDI-SRM/MS study were top ranked in the database. This is due to a significant overlap between MALDI and ESI for peptides ionization [38]. In addition, the design of the *in silico* SRM transitions set is simplified because with MALDI the precursor charge is always +1 and the systematic calculation of the fragments *m/z* is restricted to the y-ions series due to the SPITC derivatization. This strategy produced firstly a broad SRM transitions set used to locate the peptides onto the MALDI plate. That step did not necessitate a high number of data points per spot because the idea is to map the peptides location of the fractionated LC-MALDI run. Afterwards, the fine-tuning of the best SRM transitions for each peptide is performed by re-analysis of the spots containing only the relevant information.

Comparison of relative and absolute quantification accuracy between ESI- and MALDI-SRM/MS methods

In a previous work we investigated the MALDI-SRM/MS capabilities for the absolute quantification of a therapeutic mAb using a procedure based on analyte/IS area ratio calibration curve.[29] We found that the SPITC derivatization procedure does not systematically improve the limit of quantification but dramatically improve the fragmentation and provides additional SRM transitions to increase the selectivity of the measurements. Comparison between MS/MS spectra of SPITC derivatized and their native forms is available in the figure S1 of the supplementary data. The external calibration offers the advantages of an easier adjustment of the actual protein or peptide concentration to the MS response. However, its implementation for the quantification of large sets of proteins remains challenging and requires a representative biological matrix without endogenous compounds that might interfere with the proteins quantification. For instance, the quantification accuracy was assessed by spiking the yeast phosphoglycerate kinase (PGK) into an "equalized" human plasma digest, followed by the analysis on both the LC-MALDI-SRM/MS and the LC-ESI-SRM/MS platforms. The equalized human plasma generates a realistic biological matrix background free from most interference coming from shared yeast peptides sequences. With the ESI mode, the peptides were not derivatized with SPITC and the SRM transitions were directly extracted from the SRMAtlas database.

Two signature peptides were selected for the quantification of PGK, and at first glance the ESI and MALDI yielded very similar results in terms of accuracy; there is no bias assigned to MALDI (Table 1). The relative quantification, or so-called fold-change analysis, is based on the normalization of the light/heavy peptides area ratios in comparison to a fixed concentration or state point. The absolute quantification obtained via isotopic dilution [39] was based on the light/heavy peptides signal ratio multiplied by the perfectly known amount of heavy peptides actually spiked in the sample. The results of the relative quantification showed acceptable fold change accuracy (*i.e.* 100% +/- 20%, except for the peptide YHIEEEGSR at a concentration ratio of 40 in LC-ESI-SRM/MS) and precision data (*i.e.*, CV below 20%). Overall, the observed fold change results ranging from 0.5 to 40 were in accordance with the values already observed for similar proteins in yeast [11, 40].

The results of absolute quantification using isotopic dilution of labeled peptides showed a similar negative bias of 50 % independently of the peptide and ionization source, the protein concentration is actually under evaluated. This bias is probably due to the fact that synthetic peptides do not balance the digestion efficiency or issues related to solubilization and stability.

Table 1: Relative and absolute quantification of the phosphoglycerate kinase signature peptides LSELIGAR and YHIEEEGSR by LC-MALDI- or LC-ESI-SRM/MS. For the relative quantification, the concentrations ratio of 1 was used as 100%. Accuracy (%) = 100 × (calculated concentration / actual concentration).

LSELIGAR

LC-MALDI	**LC-ESI**

relative quantification

concentrations ratio	accuracy	RSD	concentrations ratio	accuracy	RSD
1.00	100%	5%	1.00	100%	6%
2.00	91%	3%	2.00	91%	16%
4.00	105%	15%	4.00	107%	15%
40.00	84%	6%	40.00	85%	9%

concentrations ratio	accuracy	RSD	concentrations ratio	accuracy	RSD
0.50	110%	5%	0.50	110%	6%
1.00	100%	3%	1.00	100%	16%
2.00	116%	15%	2.00	117%	15%
20.00	93%	6%	20.00	93%	9%

isotope dilution quantification

theorectical injection (femtomoles)	accuracy	RSD	theorectical injection (femtomoles)	accuracy	RSD
560	46%	5%	560	44%	6%
1121	42%	3%	1121	40%	16%
2242	48%	15%	2242	47%	15%
22418	39%	6%	22418	37%	9%

YHIEEEGSR

LC-MALDI			**LC-ESI**		
relative quantification					
concentrations ratio	accuracy	RSD	concentrations ratio	accuracy	RSD

1.00	100%	11%	1.00	100%	13%
2.00	91%	1%	2.00	85%	11%
4.00	95%	11%	4.00	93%	10%
40.00	83%	7%	40.00	69%	3%

concentrations ratio	accuracy	RSD	concentrations ratio	accuracy	RSD
0.50	110%	11%	0.50	118%	13%
1.00	100%	1%	1.00	100%	11%
2.00	104%	11%	2.00	110%	10%
20.00	91%	7%	20.00	81%	3%

isotope dilution quantification

theorectical injection (femtomoles)	accuracy	RSD	theorectical injection (femtomoles)	accuracy	RSD
560	49%	11%	560	49%	13%
1121	45%	1%	1121	42%	11%
2242	47%	11%	2242	46%	10%
22418	41%	7%	22418	34%	3%

Application to biological samples

As a proof-of-principle the microLC-MALDI-SRM/MS platform was used for the analysis of complex biological samples and was applied to a *S. cerevisiae* growth time course experiment in glucose medium. This experiment is similar to the work of Picotti *et al.* [11] in which 45 proteins were monitored and quantified from the glycolysis/gluconeogenesis pathway and TCA/glyoxylate cycles by LC-ESI-SRM/MS. We decided to monitor 17 proteins from these cycles (Table 2) in order to measure the enzymes concentration fold changes during the metabolic shift occurring during the transition from fermentative to aerobic respiration. In addition, two purity grades of internal standard were used: i) - an accurately quantified peptide internal standard used for both the relative and isotopic dilution quantifications performed on ten proteins, and ii) - a crude peptide internal standard used for the relative quantification of the six remaining proteins.

Relative quantification

The proteins were quantified by taking the time point at 9 hours as the reference, except for ICL1, ACH1 IDP2 and MLS1, for which the reference time point was 20 hours (Figure 3). A correction factor was applied in order to take into account the number of yeast cells disrupted in each experiment. The quantification was performed for one surrogate peptide per protein (shown in bold in Table 2), the additional surrogate peptides were used to confirm the expression profiles and were closely matching the quantitative profiles.

Table 2: Peptides quantified by LC-MALDI-SRM/MS. The peptides in bold were selected for absolute or relative quantification, the others were used as confirmatory peptides.

Gene name	$[M+H]^+$	Peptides sequences	Quantification experiment devised	Biological process	Expression fold change after diauxic shift
ALD4	1432.5	NEGATLITGGER	absolute and relative	growth on ethanol	up-regulated
	1282.5	**VAFTGSTATGR**			
CIT1	1522.8	AIGVLPQLIIDR	absolute and relative	TCA cycle	
	1970.9	**GLVWEGSVLDPEEGIR**			
	1382.7	VVPGYGHAVLR			
ENO1	1627.9	**LGANAILGVSLAASR**	absolute and relative	glycolysis / gluconeogenesis	
	1022.7	TFAEALR			
FUM1	1224.5	**YWGAQTQR**	absolute and relative	TCA cycle	
GLK1	1618.8	**GVLLAADLGGTNFR**	relative	glycolysis	
	1635.8	HALALSPLGAEGER			
ICL1	1685.8	**LFHEAVIDEIER**	relative	glyoxylate cycle	

KGD1	1417.6	**FLQLANEDPR**	relative	TCA cycle	
MDH1	1548.7 1879.8	DDLFAINASIVR **FISEVENTDPTQER**	relative	TCA cycle	
MLS1	1152.5	**YFLPEIR**	relative	glyoxylate cycle	
IDP2	1642.7	**ATDVIVPEEGELR**	relative	isocitrate metabolism	
ACH1	1588.7	**SQVVSNNPEMIR**	relative	growth on ethanol	
PGK1	1265.7 1334.6	YVLEHHPR **YHIEEEGSR**	absolute and relative	glycolysis / gluconeogenesis	
ENO2	1629.8 2070.0	LGANAILGVSMAAAR **TAGIQIVADDLTVTNPAR**	absolute and relative	glycolysis / gluconeogenesis	
PGI1	1143.6 1109.5	**AVYHVALR** EFSEQVR	absolute and relative	glycolysis / gluconeogenesis	stationary-regulated
HXK2	1073.4 1575.5	LSELIGAR **TGHIAADGSVYNR**	absolute and relative	glycolysis	
PYK1	1037.6 1442.7	**FSHLYR** TNNPETLVALR	absolute and relative	glycolysis	
PDC1	1590.6	**NPVILADACCSR**	absolute and relative	glucose fermentation	

Sample fractionation on the MALDI plate was limited to a 6.4 minutes range from the 15 minutes gradient LC run. This provides a coarse but fast fractionation step of the complex biological sample compared to LC-ESI-SRM/MS where 30 to 60 minutes chromatographic gradients are of common practice for targeted proteomic studies or much longer for unsupervised assays [41]. The total acquisition time with the MALDI triple quadrupole was of 28.8 minutes per sample. However, there is still space for optimizing the acquisition time since only 40% of the column length, for a 384 wells MALDI plate, is actually dedicated to the sample (the remaining 60% representing the spaces between the spots). But the current version of our MALDI source plate controller cannot adapt its speed to go faster in-between the spots in order to avoid spending time rastering the region of the MALDI plate without relevant MS information.

Figure 3 shows that the relative proteins quantification profiles are similar to those published by Picotti *et al.* [11]. Firstly, we observed a group of proteins (ENO2, HXK2, PGI1, PGK1, PYK1 and PDC1) with no manifest changes in the expression level during growth (fold change < 2). These enzymes are involves in the glycolysis pathway and alcoholic fermentation (case of the pyruvate decarboxylase 1, PDC1). A second group displays an over-expression ranging from 3 to 33 fold after the diauxic shift. This change includes the TCA cycle enzymes (CIT1, MDH1, FUM1, KGD1) and it is due to the activation of the aerobic respiration metabolism. The aldehyde dehydrogenase 4 (ALD4) is glucose repressed and implicated in the oxidation of the ethanol in acetate before its introduction in the TCA cycle. ALD4 shows the highest level (33 fold) after 50 hrs of growth. Its induction coincides with the end of the glucose consumption. The isoform GLK1 which is de-repressed after the switch to a non-fermentable carbon source [42] is induced by 8 fold. The ENO1 which is repressed by the glucose shows a maximum fold change of 6. A third group was formed by the ACH1, implicated in the ethanol

conversion to acetyl-CoA via acetate and by ILC1/MLS1, involved in the glyoxylate cycle show an induction of 2.5 fold between 20 hrs and 50 hrs of growth. The glyoxylate cycle is implicated in the simple carbon source consumption and TCA cycle intermediates regeneration.

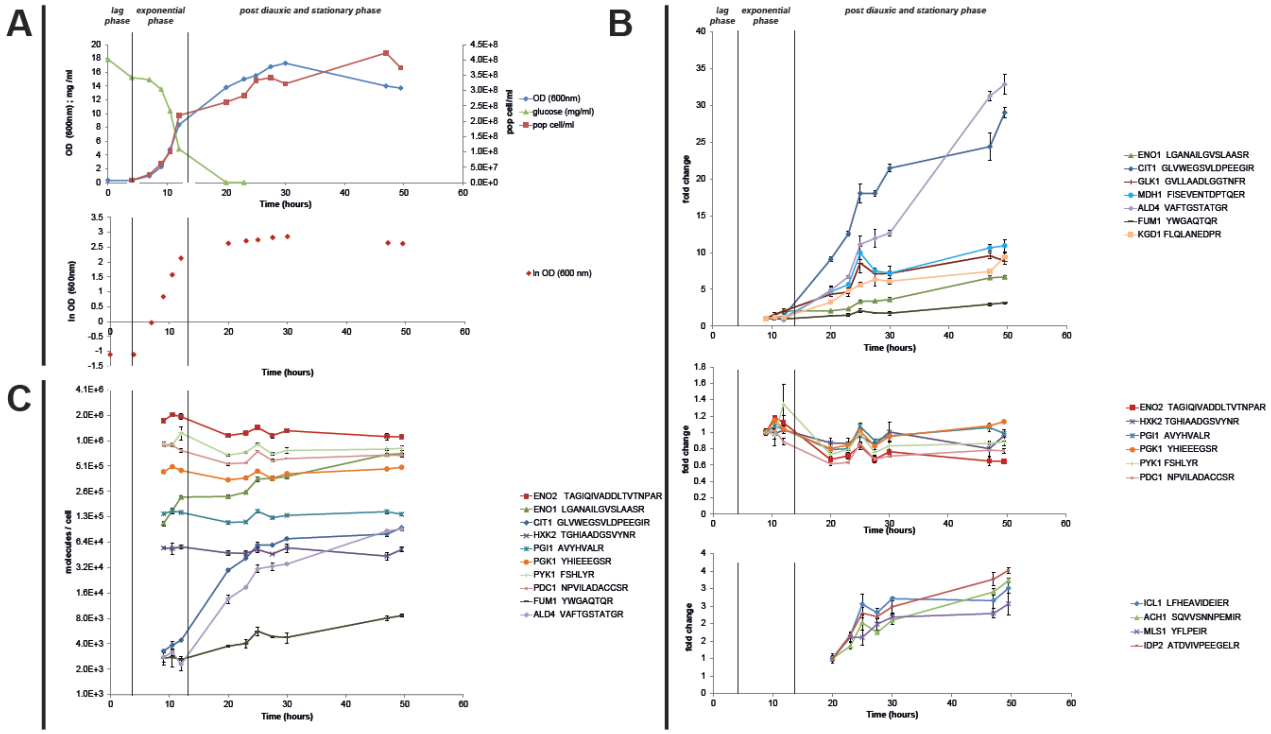


Figure 3: *Time course monitoring of S. cerevisiae proteins involved in carbon metabolism (errors bars represent the standard error on the 3 technical replicates) -* $std\ error = \frac{std\ deviation}{\sqrt{n}}$.

A) Yeast growth on glucose medium monitored by the population in the medium, OD at 600nm and glucose consumption. The OD is also plotted on a neperian logarithmic scale in order to evaluate the growth phases. B) Relative quantification of targeted proteins. The fold changes are calculated against the 9 hours point (except ICL1, ACH1, MLS1 and IPD2 for which the starting point is 20 hours). C) Absolute quantification using isotope dilution. Results are expressed in molecules/cell with log_2 *scale.*

Absolute quantification by isotopic dilution

The precise quantification by isotopic dilution of the 19 peptides was based on the amount of spiked peptides, which is determined by the supplier. However, the use of synthetic peptides has some well-known drawbacks. In the case of multimeric proteins, the measured concentrations correspond to the protein subunit concentration and not to the active complex. Moreover the quantification via peptides standard does not account for the tryptic digestion efficiency. We demonstrated that tryptic peptides from the same protein can follow several digestion kinetics [43]. Therefore, when several surrogate peptides are quantified for a protein, only the peptide giving the highest protein concentration will be

retained for the protein quantification result. This should minimize the bias from the digestion efficiency than can lead to protein quantification under estimation.
Table 3 compares the results obtained by LC-MALDI-SRM/MS along the time course to affinity-based results from the literature [44] that are obtained from a single time point. Except for ENO2 and PDC1, the results do not deviate from the literature values more than a factor of 3.

Table 3: Absolute quantification of peptides using isotopic dilution. Data are expressed as the average and the range (i.e., min and max values) along the growth time course.

[a] Values given from the literature (Ghaemmaghami *et al.*[44])

Protein	Average (molecules/cell)	Min (molecules/cell)	Max (molecules/cell)	Literature values[a] (molecules/cell)
ENO2	1'452'000	1'137'000	2'082'000	3'000
ENO1	348'000	107'000	715'000	77'000
CIT1	45'000	3'000	95'000	-
HXK2	51'000	44'000	56'000	114'000
PGI1	135'000	110'000	150'000	92'000
PGK1	431'000	351'000	502'000	314'000
PYK1	861'000	685'000	1'262'000	291'000
PDC1	703'000	543'000	902'000	9'000
FUM1	5'000	3'000	9'000	7'000
ALD4	32'000	2'000	92'000	22'000

Conclusions

A novel LC-MALDI-MS/MS platform was developed for the quantitative analysis of peptides in biological matrices. From an instrumental perspective the system is based on the unique combination of a fast rastering MALDI source using a high frequency laser (1000 Hz) for ions generation with a triple quadrupole linear ion trap mass spectrometer operated in the selected reaction monitoring mode for high sensitivity. The time devoted to the MS acquisition, ranging from 425 ms to several minutes per spot, fits the analytical needs of the high throughput analysis of either a few targeted compounds or relatively complex proteome studies. As only a small amount of the sample present in one spot (about 8%) is consumed during the analysis, thus each spot can be reanalyzed several times under different conditions including SRM mode, enhanced product ion mode or MS^3 mode. The MALDI plates can easily be shipped and stored; therefore samples from different laboratories can be analyzed on a single mass spectrometer in a high throughput regime. Fragmentation of singly charged peptides was found to be peptide sequence dependent and derivatization by sulfonation of the N-terminal α-amino group significantly enhanced the fragmentation efficiency under low energy collision induced dissociation. The applicability of microscale LC-MALDI-SRM/MS for the quantitative analysis of selected pathways in yeast lysate was demonstrated. As the chromatographic step is decoupled from the MS detection and still remains the time limiting step, one could envisage parallel LC systems for increasing the throughput in combination with orthogonal pre-fractionation techniques, such as strong cation exchange chromatography to optimize selectivity.

The LC-MALDI platform appears to be complementary to the conventional LC-ESI hyphenation for SRM based experiments and perfectly suited for the high throughput quantification of small peptides sets. Ultimately a specific sample preparation such as immuno-capture and concentration of the targeted peptides would allow avoiding the liquid chromatographic fractionation step for an efficient analysis of large cohorts of samples as required for biomarkers validation.

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Supporting information

Peptides quantification by liquid chromatography with Matrix-Assisted Laser Desorption/Ionization and Selected Reaction Monitoring detection

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Supplemental Discussion

Chemically assisted fragmentation of SPITC-derivatized peptides

The singly charged peptides, typically produced by MALDI sources, generally display a poor fragmentation efficiency under low energy CID [1] and post source decay [2] on the contrary to their multiply charged counterparts generated by electrospray. However, the MS/MS fragmentation efficiency of singly charged peptides under low energy CID can be greatly improved by chemically assisted fragmentation. In this study, the 4-sulfophenylisothiocyanate was used as derivative agent that reacts with the primary amino group at the N-terminus of peptides and generates a mass increase of 215 u. Because the SPITC may also react with the amino group of the lysine side chain, arginine-ending peptides are preferred; however, lysine-ending peptides can be converted into homoarginine by a fast (5 min) guanidination reaction [3]. The sulfonic acid group holds a strong negative charge and a second proton is required in order to produce a singly charged peptide. Because the tryptic peptides are ended by a basic amino acid residue with a substantial proton affinity, one of the two protons tends to be sequestered at the C-terminus of the sulfonated peptide. The second proton is more freely delocalized along the peptide backbone and consequently promotes the fragmentation [4]. Figure S1 illustrates the MS/MS fragmentation of both native and SPITC derivatized peptides acquired with the MALDI-QqQ_{LIT} instrument. Native peptides MS/MS spectra display weak fragmentation efficiency with generally few assignable fragments and an overall lower signal to noise. The MS/MS spectra of native peptides are more complex than their sulfonated counterparts; they contain a mix of b- and y-ions series, with losses of water and ammonia. On the contrary, MS/MS spectra of SPITC derivatized peptides are characterized by a clear y-ions series with high signal to noise. However, the b-ion fragments are nearly not detected because of the negative charge on the SPITC moiety. Therefore, the fragmentation pattern of sulfonated peptides is more suitable for SRM experiments since it efficiently produces predictable fragments ions (y-ion fragments). This particular aspect of the chemically assisted fragmentation acts as a selectivity enhancer because alternative SRM transitions are available in case of interferences.

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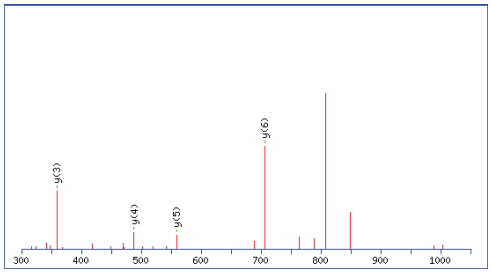
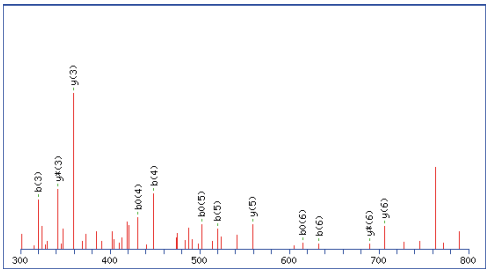
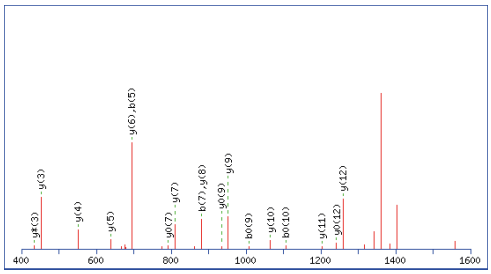
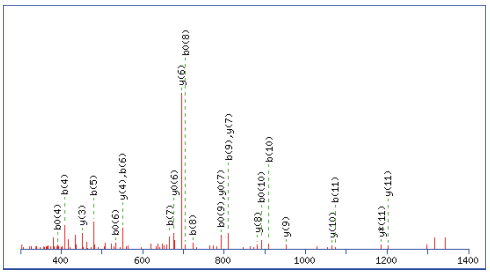
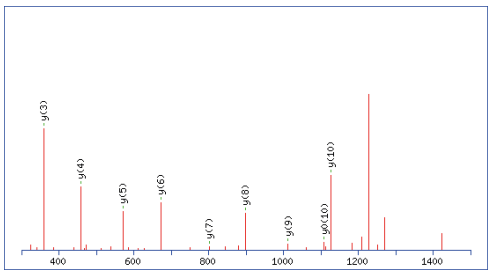
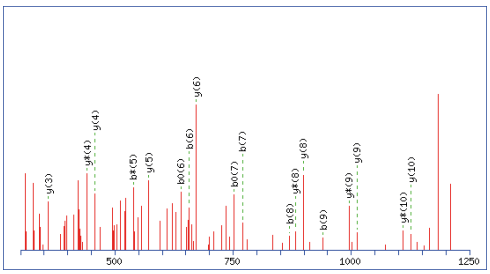
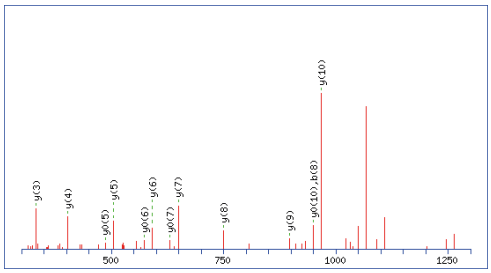
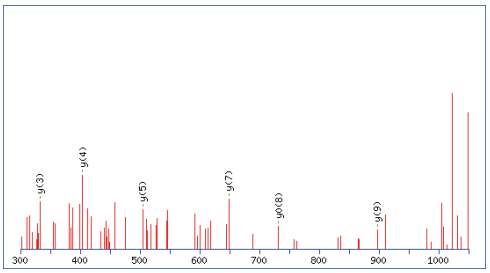
TFAEALR		
TGHIAADGSVYNR		
TNNPETLVALR		
VAFTGSTATGR		

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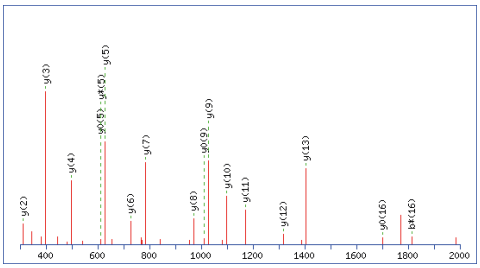
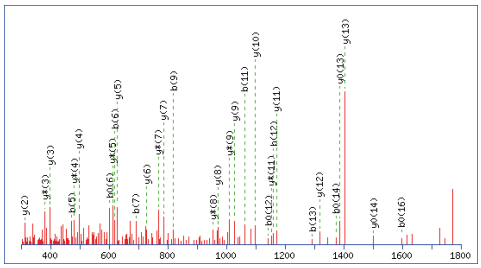
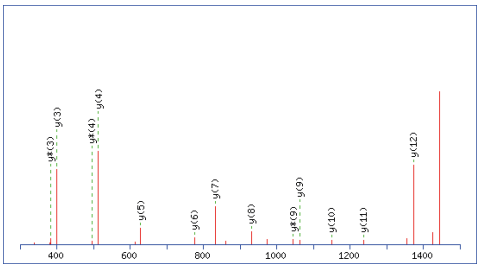
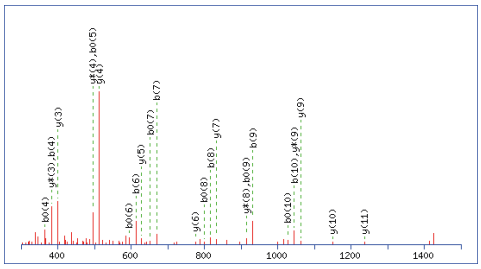
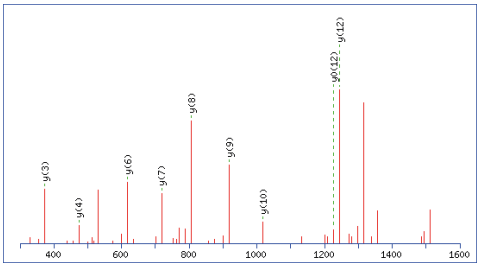
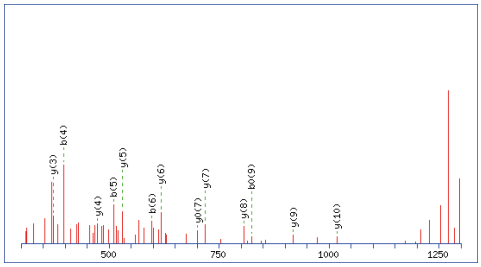
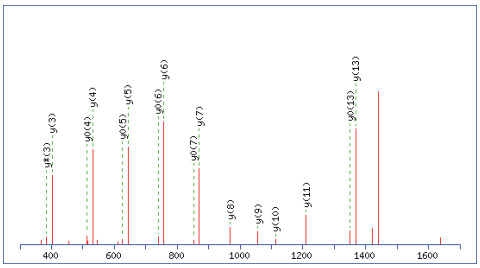
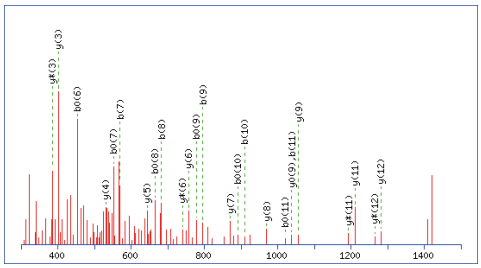
AAQDSFAAGWGVMVSHR		
AHSSMVGFDLPQR		
AIIVLSTSGTTPR		
ASAPGSVILLENLR		

SPITC

Native

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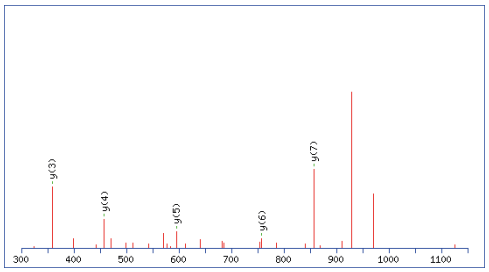

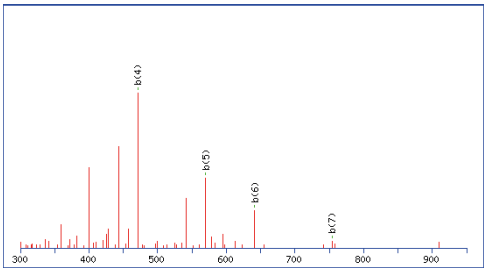

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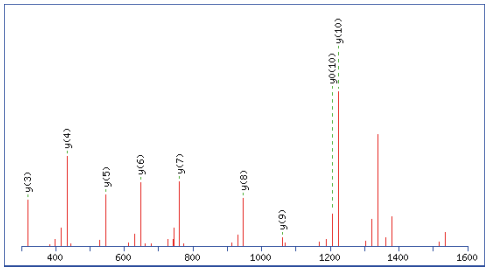

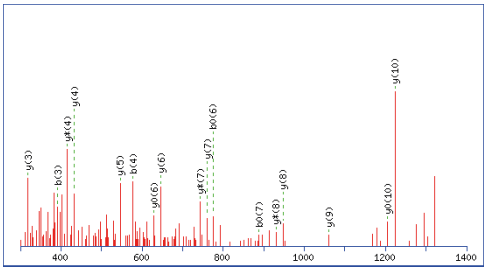

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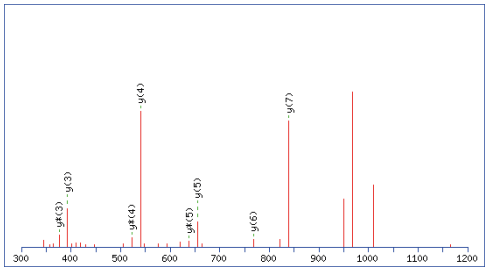

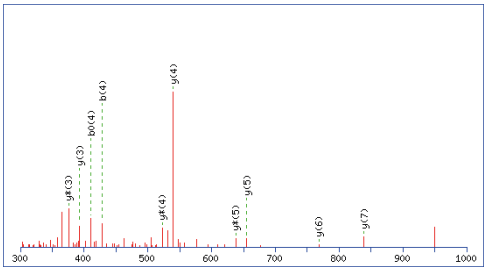

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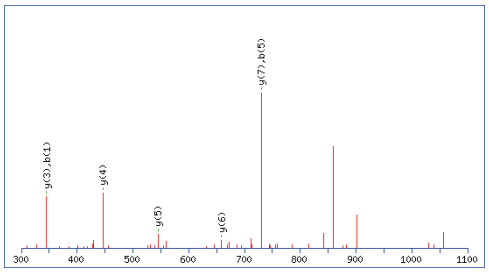

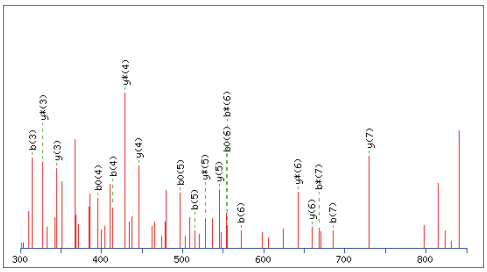

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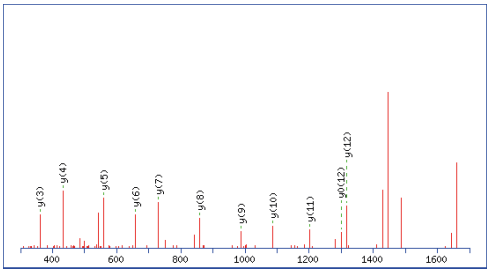
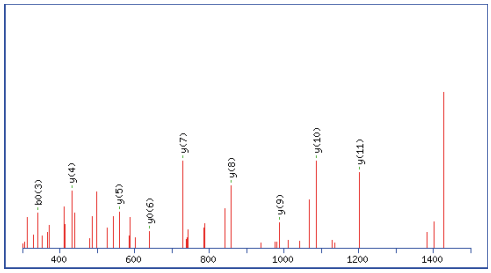
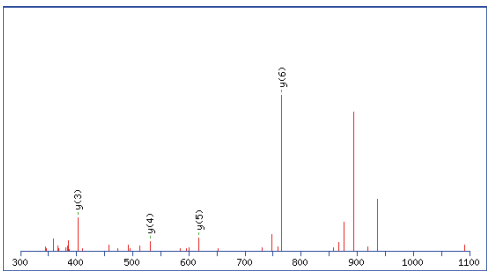
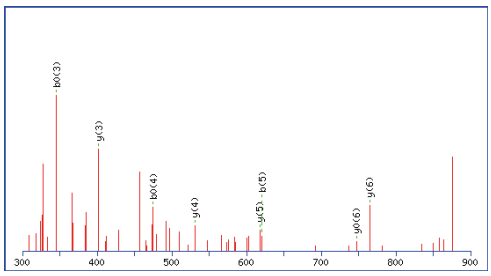
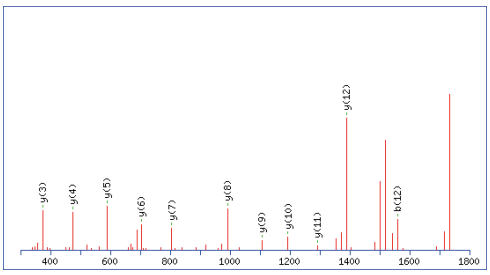
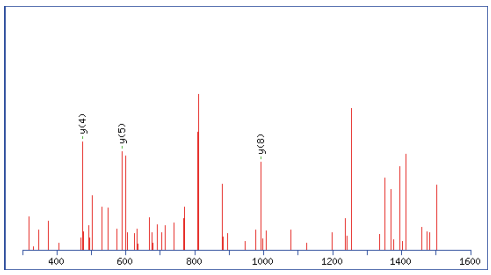
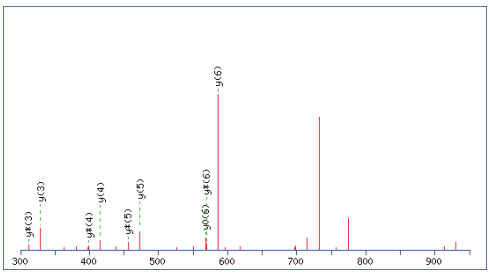
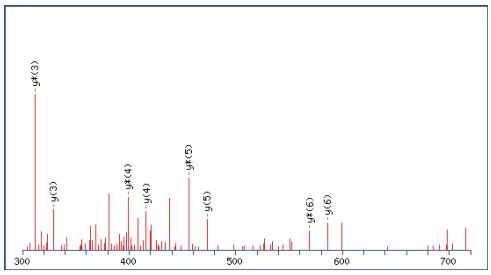
EDDVEEAVQAADR		
EFSEQVR		
EPVSDWTDDVEAR		
FLGSGPR		

SPITC

Native

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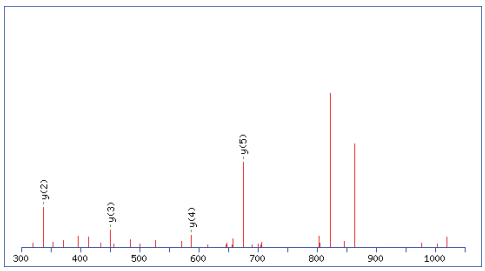

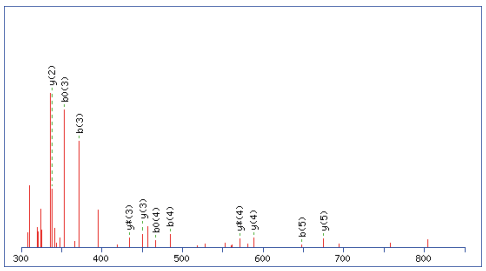

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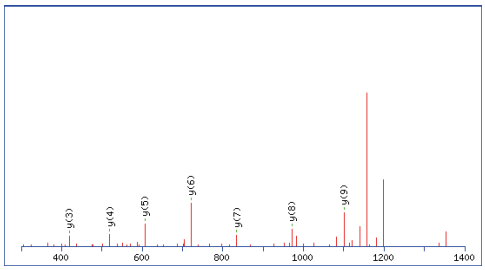

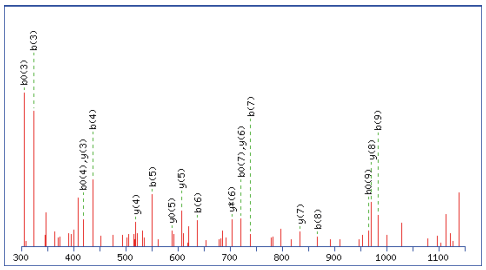

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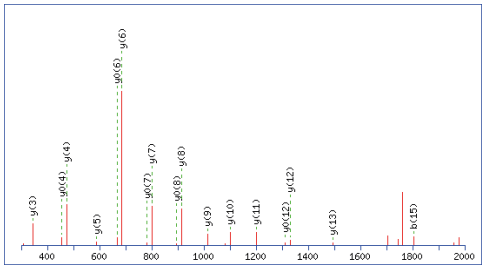

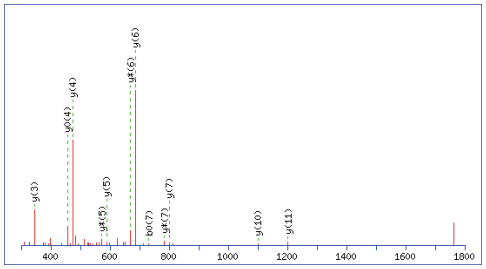

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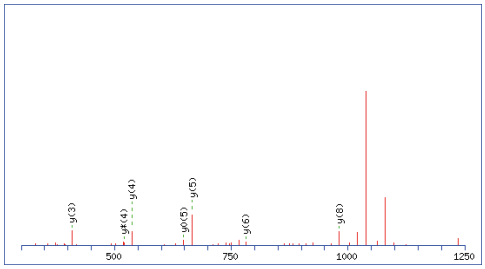

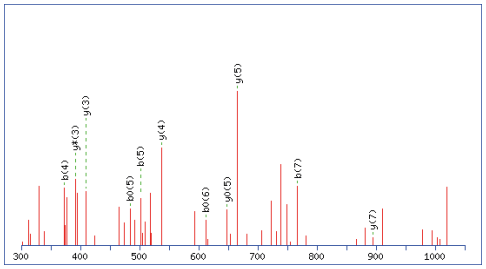

SPITC

Native

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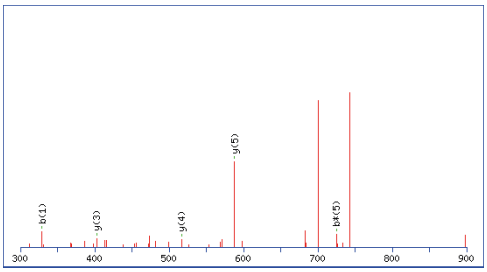

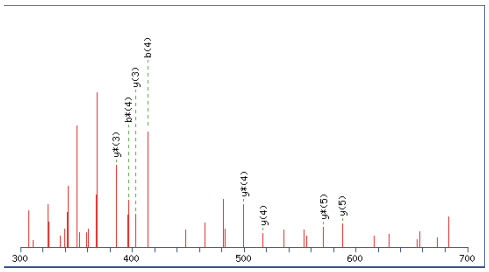

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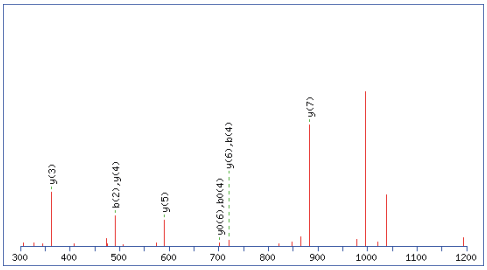

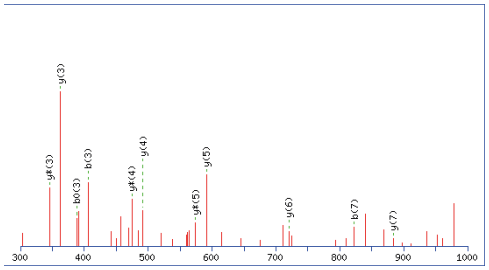

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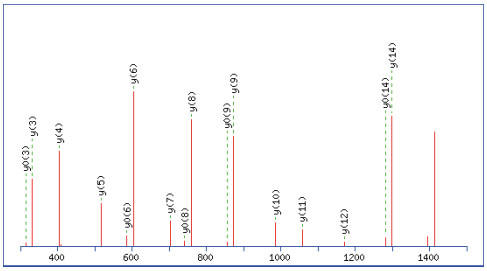

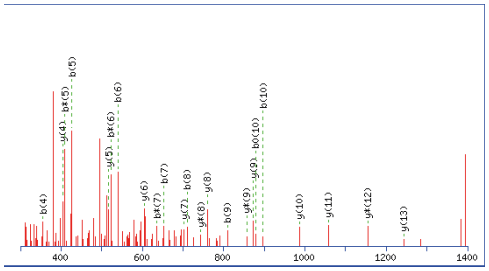

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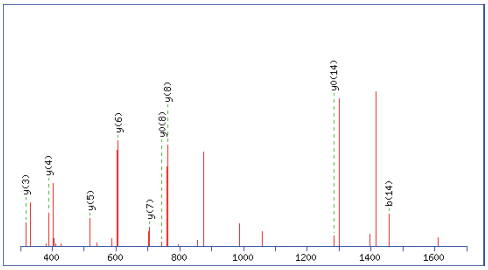
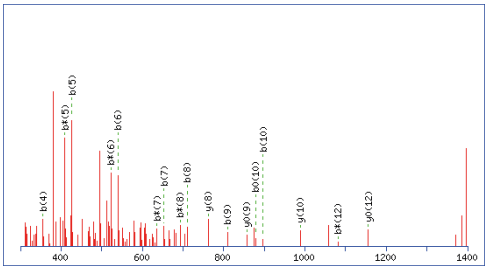
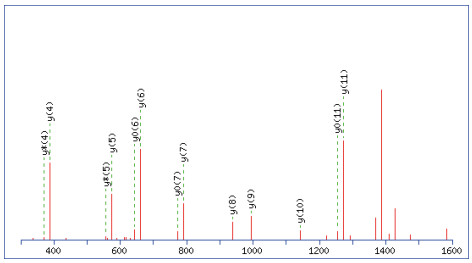
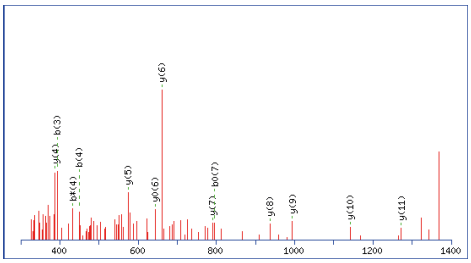
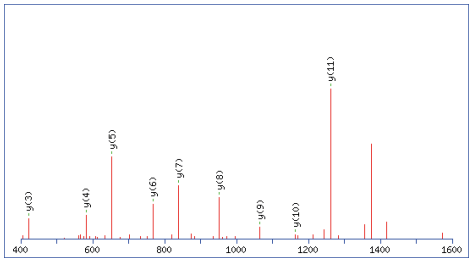
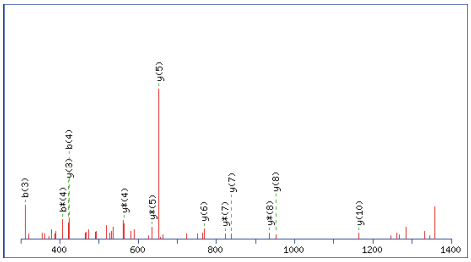
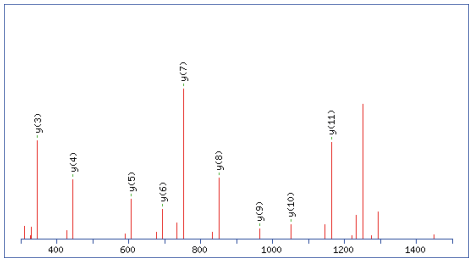
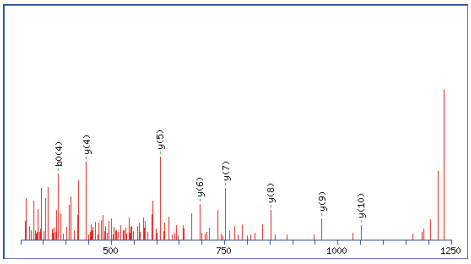
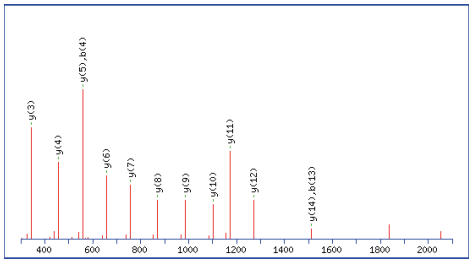
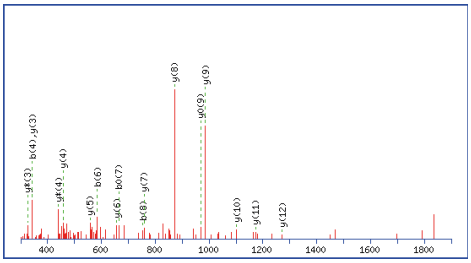
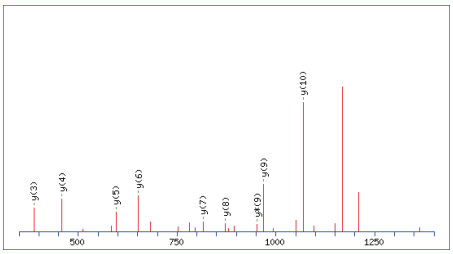
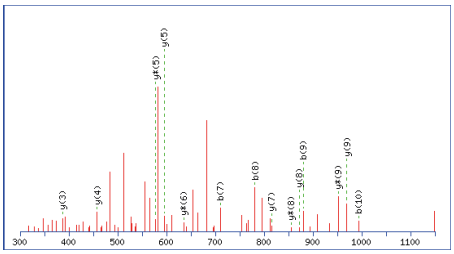
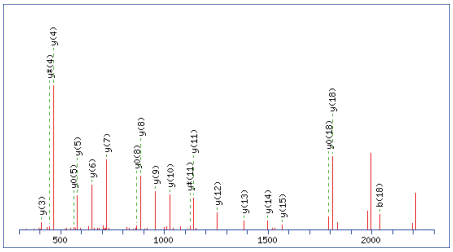
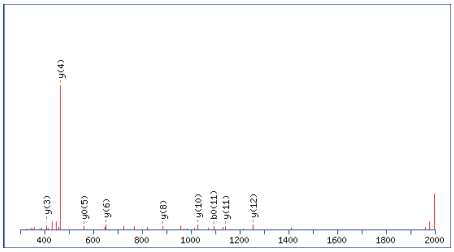
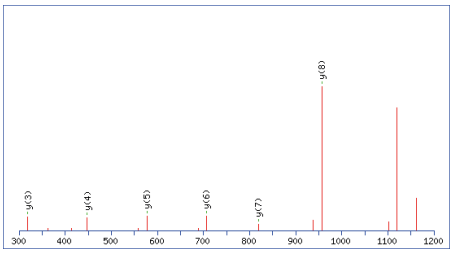
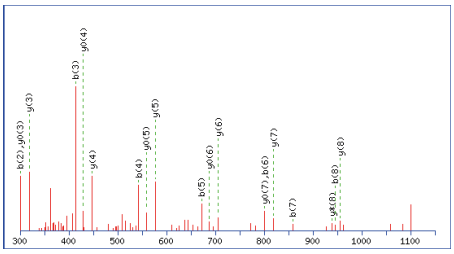
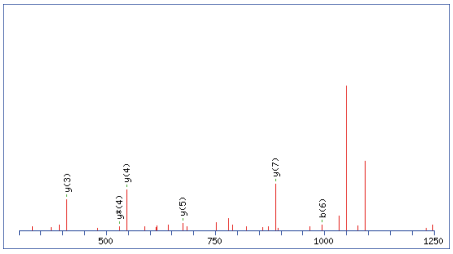
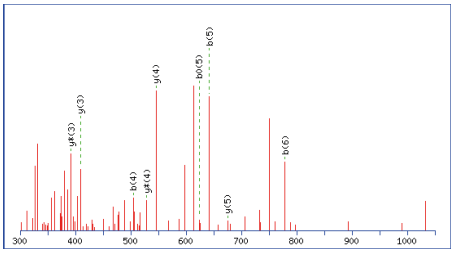
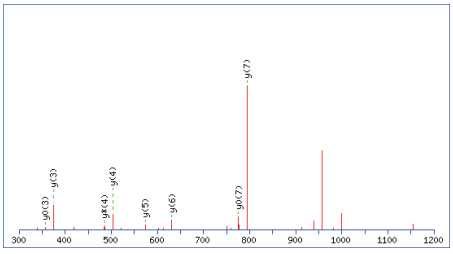
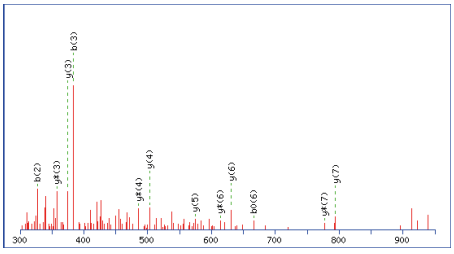




	SPITC	Native
NMFGFESWVGGR		
NPVILADACCSR		
SISIVGSYVGNR		
TAGIQIVADDLTVTNPAR		

	SPITC	Native
VVPGYGHAVLR		
WAGNANELNAAYAADGYAR		
YHIEEEGSR		
YVLEHHPR		
YYGAQTVR		

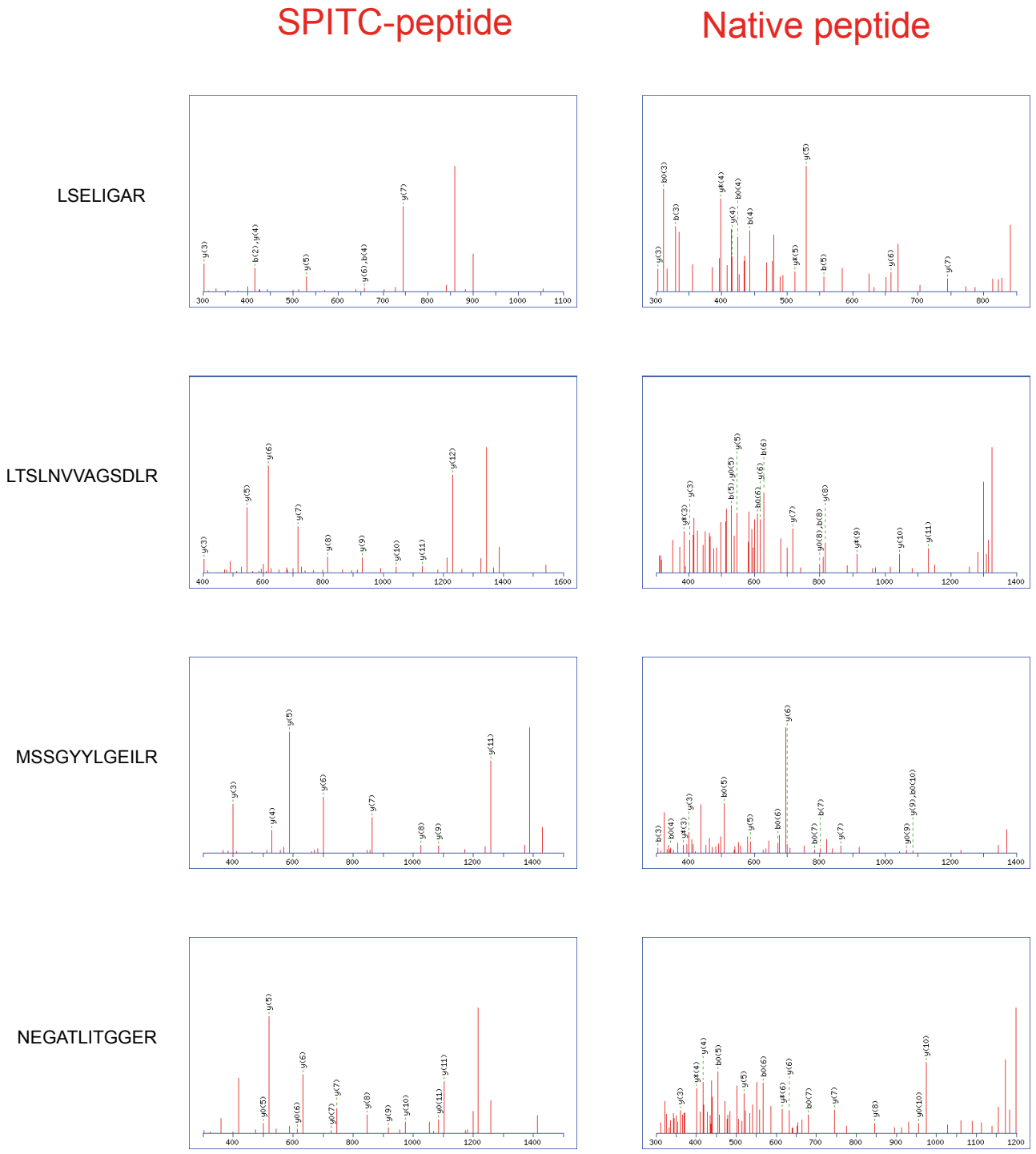


Figure S1: Reconstructed MS/MS spectra of 37 peptides acquired on the MALDI-QqQ$_{LIT}$, both as native and SPITC derivatized peptides. Precursor ions were removed by software.

Optimization of the collision energy for SPITC-derivatized peptides

Historically, the N-terminal sulfonation of peptides was designated for post-source decay (PSD) experiments on MALDI-TOF instruments. Nowadays, few applications of chemically assisted fragmentation were published with MALDI-TOF/TOF (high energy CID) [5] or ion trap (low energy CID).[6] There is a lack of data in the literature regarding the optimal collision energy (CE) for fragmenting singly charged sulfonated peptides on triple quadrupole or QqTOF instruments. In a previous work [7] the CE was optimized manually for each transition, this is acceptable for the monitoring of few peptides but for the purpose of developing extensive SRM transitions sets, a generic formula linking the optimal CE value with the peptides *m/z* is essential. Therefore, 261 observable SRM transitions were selected from 37 derivatized peptides (with precursor ions *m/z* ranging from 916.5 to 2212.8 u) and the collision energy was tuned for every SRM transition by steps of 10 eV (Table S1). The optimal CE value for each peptide was calculated by averaging the best CE of its SRM transitions. These calculated CE values were plotted against the precursor ions *m/z* (Figure S2). The following linear regression equation was obtained: CE (eV) = 17.5 + 0.044 × *m/z* with a coefficient of determination (R2) of 0.92. This formula was used for this present study.

Table S1: Optimized CE for every SRM transitions of 37 peptides sulfonated peptides. The optimal CE for a peptide consists in the CE average of the individual SRM transitions.

peptide	Q1	Q3	fragment	Optimal CE (eV)	averaged CE (eV)
SISIVGSYVGNR	1466.7	346.2	y3	75	78
		445.3	y4	75	
		608.3	y5	75	
		695.4	y6	85	
		752.4	y7	75	
		851.4	y8	75	
		964.5	y9	85	

		1051.6	y10	85	
		1164.6	y11	75	
EALDFFAR	1183.6	393.2	y3	60	68
		540.3	y4	80	
		655.3	y5	70	
		768.4	y6	70	
		839.4	y7	60	
NEGATLITGGER	1432.5	418.2	y4	75	78
		519.3	y5	75	
		632.3	y6	65	
		745.4	y7	85	
		846.5	y8	75	
		974.5	y10	95	
		1103.6	y11	75	
VAFTGSTATGR	1282.5	505.3	y5	75	70
		592.3	y6	65	
		649.3	y7	75	
		750.4	y8	65	
		897.4	y9	65	
		968.5	y10	75	
EDDVEEAVQAADR	1661.5	560.3	y5	95	96
		659.4	y6	85	
		730.4	y7	115	
		859.4	y8	95	
		988.5	y9	95	
		1087.5	y10	95	
		1202.6	y11	105	
		1317.6	y12	85	
YYGAQTVR	1172.5	375.2	y3	70	68

		503.3	y4	70	
		574.3	y5	70	
		631.5	y6	70	
		794.4	y7	60	
FLGSGPR	948.5	329.2	y3	60	58
		416.2	y4	60	
		473.3	y5	60	
		586.3	y6	50	
IANDIR	916.5	403.2	y3	50	50
		517.3	y4	50	
		588.3	y5	50	
VVPGYGHAVLR	1382.7	387.3	y3	80	84
		458.3	y4	80	
		595.4	y5	90	
		652.4	y6	80	
		815.5	y7	90	
		872.5	y8	90	
		969.5	y9	80	
		1068.6	y10	80	
DYIWNTLNSGR	1553.6	319.2	y3	80	84
		433.2	y4	80	
		546.3	y5	90	
		647.4	y6	90	
		761.4	y7	80	
		947.5	y8	80	
		1060.6	y9	90	
		1223.6	y10	80	
GLVYETSVLDPDEGIR	1977.7	345.2	y3	120	103
		474.3	y4	120	

		589.3	y5	120	
		686.4	y6	120	
		801.4	y7	80	
		914.5	y8	90	
		1013.5	y9	100	
		1100.6	y10	100	
		1201.6	y11	90	
		1330.7	y12	90	
EANVTGLR	1074.5	345.2	y3	60	62
		446.3	y4	60	
		545.3	y5	60	
		659.4	y6	70	
		730.4	y7	60	
GEHINSTEDR	1372.5	419.2	y3	80	80
		520.2	y4	80	
		607.3	y5	80	
		721.3	y6	80	
		834.4	y7	80	
		971.5	y8	90	
		1100.5	y9	70	
NMFGFESWVGGR	1601.6	388.2	y4	80	86
		574.3	y5	100	
		661.3	y6	90	
		790.4	y7	90	
		937.5	y8	90	
		994.5	y9	90	
		1141.5	y10	80	
		1272.6	y11	70	
AVYHVALR	1143.6	359.2	y3	70	70

		458.3	y4	70	
		595.4	y5	70	
		758.4	y6	70	
		857.5	y7	70	
EFSEQVR	1109.5	402.3	y3	70	65
		531.3	y4	70	
		618.3	y5	60	
		765.4	y6	60	
GSIDEQHPR	1253.6	409.2	y3	75	81
		537.3	y4	85	
		666.3	y5	85	
		781.4	y6	85	
		981.5	y8	75	
NPVILADACCSR	1590.6	422.2	y3	70	83
		582.2	y4	90	
		653.3	y5	100	
		768.3	y6	80	
		839.3	y7	80	
		952.4	y8	80	
		1065.5	y9	80	
		1261.0	y11	80	
WAGNANELNAAYAADGYAR	2212.8	466.2	y4	120	106
		581.3	y5	90	
		652.3	y6	110	
		723.3	y7	110	
		886.4	y8	110	
		957.4	y9	100	
		1028.5	y10	120	
		1142.5	y11	110	

		1255.6	y12	120	
		1384.7	y13	100	
		1498.7	y14	80	
		1811.8	y18	100	
IYEVEGMR	1211.6	363.2	y3	65	67
		492.2	y4	65	
		591.3	y5	75	
		720.3	y6	65	
		883.4	y7	65	
AIIVLSTSGTTPR	1530.7	373.2	y3	70	83
		474.3	y4	80	
		531.3	y5	100	
		618.3	y6	90	
		719.4	y7	80	
		806.4	y8	80	
		919.5	y9	80	
		1018.6	y10	90	
		1244.7	y12	80	
EPVSDWTDDVEAR	1733.6	375.2	y3	90	96
		474.3	y4	110	
		589.3	y5	100	
		704.3	y6	80	
		805.4	y7	90	
		991.5	y8	110	
		1106.5	y9	100	
		1193.5	y10	90	
		1389.6	y12	90	
FSHLYR	1037.6	338.2	y2	65	63
		451.3	y3	65	

		588.3	y4	65	
		675.4	y5	55	
TNNPETLVALR	1442.7	359.2	y3	75	77
		458.3	y4	75	
		571.4	y5	75	
		672.4	y6	75	
		898.5	y8	85	
		1126.6	y10	75	
LTSLNVVAGSDLR	1559.7	403.2	y3	80	84
		490.3	y4	90	
		547.3	y5	80	
		618.3	y6	80	
		717.4	y7	80	
		816.5	y8	90	
		930.5	y9	80	
		1130.6	y11	100	
		1231.7	y12	80	
YVLEHHPR	1265.7	409.2	y3	75	78
		546.3	y4	85	
		887.5	y7	75	
YHIEEEGSR	1334.6	319.2	y3	90	83
		448.2	y4	90	
		577.3	y5	90	
		706.3	y6	80	
		819.4	y7	80	
		956.4	y8	70	
ASAPGSVILLENLR	1654.8	402.2	y3	85	89
		531.3	y4	85	
		644.4	y5	95	

		757.5	y6	75	
		870.5	y7	85	
		969.6	y8	95	
		1056.6	y9	95	
		1113.7	y10	95	
		1210.7	y11	95	
		1368.8	y13	85	
AHSSMVGFDLPQR	1659.7	400.2	y3	85	95
		513.3	y4	105	
		628.3	y5	95	
		832.4	y7	85	
		931.5	y8	95	
		1373.7	y12	105	
LSELIGAR	1073.4	303.2	y3	60	63
		416.3	y4	60	
		529.3	y5	70	
		745.4	y7	60	
TGHIAADGSVYNR	1575.5	452.2	y3	80	91
		551.3	y4	100	
		695.3	y6	100	
		810.4	y7	90	
		881.4	y8	90	
		952.4	y9	90	
		1065.5	y10	90	
		1259.6	y12	90	
MSSGYYLGEILR	1603.5	401.3	y3	80	88
		530.3	y4	90	
		587.4	y5	80	
		700.4	y6	90	

		863.5	y7	90	
		1026.6	y8	90	
		1083.6	y9	100	
		1257.6	y11	80	
TFAEALR	1022.7	359.2	y3	65	63
		488.3	y4	65	
		559.3	y5	65	
		706.4	y6	55	
LGANAILGVSLAASR	1627.9	333.2	y3	85	90
		404.2	y4	85	
		517.3	y5	95	
		604.3	y6	95	
		703.4	y7	75	
		760.4	y8	85	
		873.5	y9	95	
		986.6	y10	95	
		1057.6	y11	95	
		1171.7	y12	95	
		1299.7	y14	95	
AAQDSFAAGWGVMVSHR	2004.8	399.2	y3	110	112
		498.3	y4	110	
		629.3	y5	110	
		728.4	y6	110	
		785.4	y7	110	
		971.5	y8	120	
		1028.5	y9	110	
		1099.5	y10	110	
		1170.6	y11	100	
		1317.7	y12	120	

		1404.7	y13	120	
		1519.7	y14	110	
LGANAILGVSMAAAR	1629.8	317.2	y3	75	89
		388.2	y4	95	
		519.3	y5	105	
		606.3	y6	75	
		705.4	y7	85	
		762.4	y8	85	
		875.5	y9	95	
		988.6	y10	95	
TAGIQIVADDLTVTNPAR	2070.0	343.2	y3	100	103
		457.3	y4	100	
		558.3	y5	100	
		657.4	y6	100	
		758.4	y7	100	
		871.5	y8	120	
		986.5	y9	120	
		1101.6	y10	90	
		1172.6	y11	100	
		1271.7	y12	100	
		1384.7	y13	110	
		1512.8	y14	100	

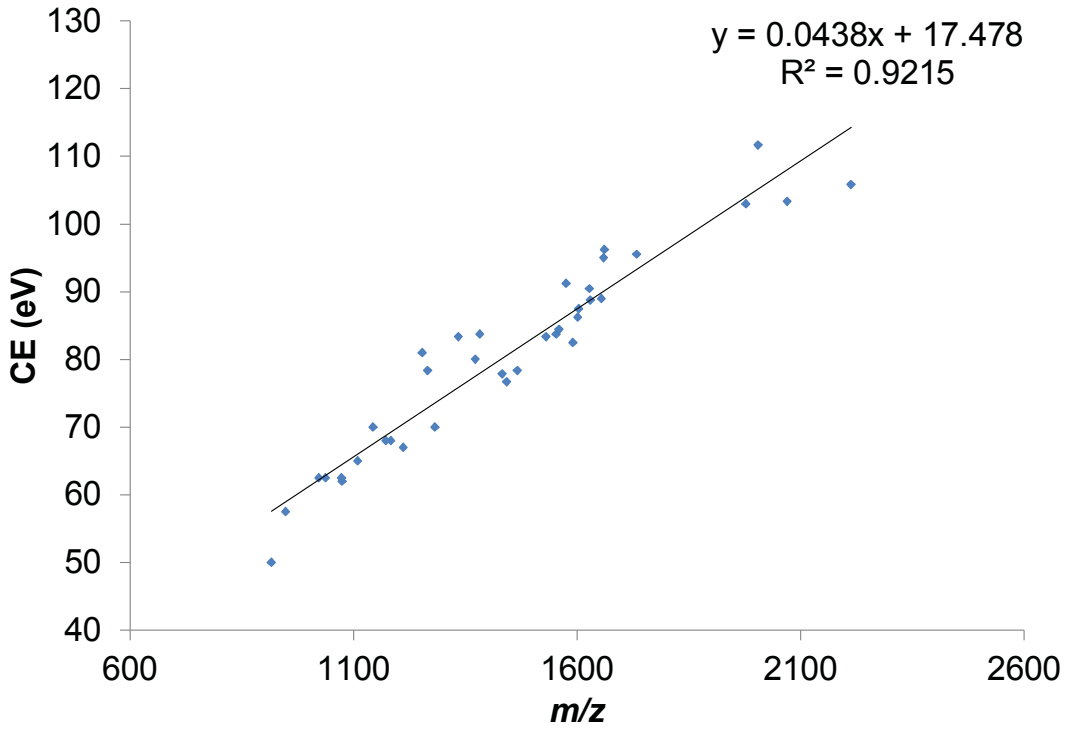


Figure S2: Plot of the optimal CE in function of the SPITC-derivatized peptide precursor ion *m/z*.

Sample clean up prior LC-MALDI-SRM/MS analysis

We routinely derivatized 50 µg of a protein tryptic digest with 1 mg of SPITC technical grade reagent. It represents a theoretical reagent excess of 100-fold when the average mass of yeast tryptic peptides is estimated to 1220 Da [8]. After the derivatization step, samples contain an excess of SPITC reagent, as well as impurities and by-products from the SPITC synthesis (ions with sulfur isotopic patterns) that produce severe contamination of the reverse phase chromatography. It is common to perform a sample clean up step after SPITC derivatization, usually by using a C18 solid phase extraction (SPE) [9, 10].

However, these contaminants display a significant retention onto the analytical column, and neither C18, nor column-switching was suitable to efficiently remove these contaminants from the derivatized peptides. The excess of SPITC reagent impedes the crystallization process with CHCA (i.e. bigger crystals that do not disintegrate under laser irradiation) and can generate ionization suppression. This point was addressed using a size exclusion sample clean up on homemade single use devices packed with Sephadex G10 (Figure S3). Fractions 2 and 3 were merged for the analysis.

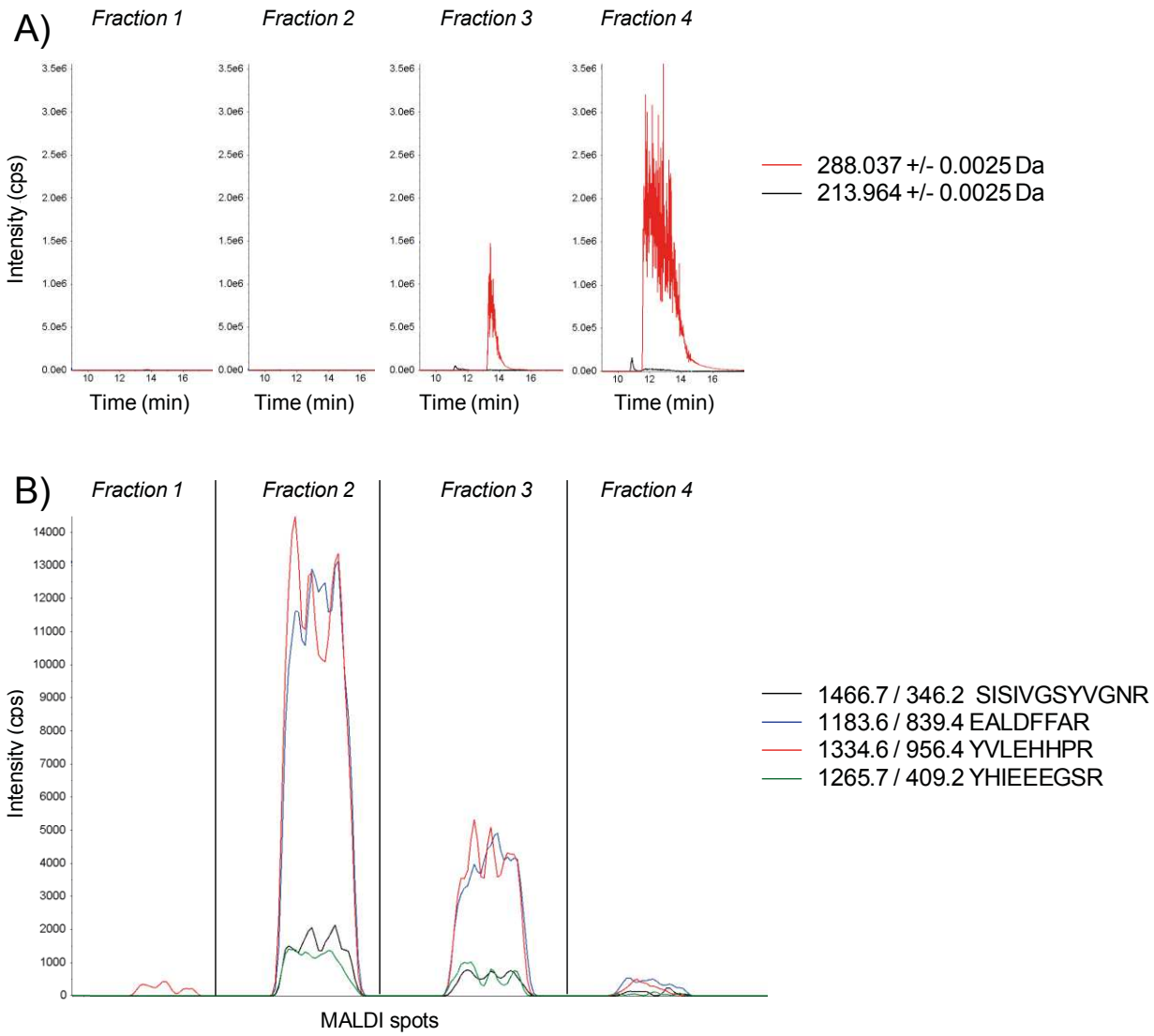


Figure S3: Efficiency of the gel filtration clean-up of a tryptic digest derivatized by SPITC. The first four gel filtration eluted fractions were analyzed by:

- (A) a LC-ESI-QqTOF in negative mode, with a column-switching set up, in order to monitor the main contaminants distribution.

- (B) a MALDI-SRM/MS in positive mode in order to monitored the peptides distribution in the fractions.

Sensitivity of the MALDI-SRM/MS platform

The size of the laser beam on the sample spot is not the real limitation of the MALDI-MS sensitivity. Increasing the laser beam size will increase the total number of ions generated but with less efficiency. There is a non-linear dependence between laser beam size and ions yield [11]. The laser beam area of our particular set up is 6.28e-4 cm^2, it represents 2.8% of a spot surface (1700 µm diameter). During the acquisition the laser pass through the MALDI spot linearly (Figure S4 B) in order to increase the acquisition time and balance the inhomogeneous distribution of the sample in the spot. The response factor is dependent of the density of analytes (molecules/surface) on the sample spot surface until analytes suppression effect occurs [12]. The analytes density on the spot is set by the concentrations in the post LC column flow, the volume of the droplet collected onto the MALDI plate and the spot surface. According to these considerations the response factor of an LC-MALDI-MS can be enhanced by:

- a reduction of the spot surface
- an increased volume of the collected droplets
- an increased amount of the sample injected onto the LC column

Smaller spots are not desirable because for a constant MS duty cycle the acquisition speed must be reduced in order to keep the data sampling constant. The sampling rate of the LC flow onto the MALDI plate is also a critical point; ideally it must be long enough to pool a whole chromatographic peak but in the other hand short enough to keep the LC fractionation effective. The LC-MALDI fractionation of peptides is usually performed with a nanoscale LC set up, but here a micro-LC configuration was selected because of the higher loading capacity of the 1 mm ID analytical column compared to 75 µm ID nanoscale columns. However, LC columns with higher ID require higher flow rate (typically 50 µl/min) that can lead to a detrimental chromatographic peak splitting over the spots. This issue was adressed by using hydrophobic MALDI plates allowing a collection volume of 4 µl/spot at a sampling rate of 4s/spot (Figure S4 A). The hydrophobic MALDI plates also offer the advantage of keep the spot surface constant during evaporation despite a higher droplet volume. The post-column MALDI matrix infusion was performed with a highly concentrated solution at a low flow rate (10 µl/min) in order to limit the

dilution of the chromatographic flow. Using these parameters, the chromatographic peaks were split over 3 to 5 MALDI spots (see Figure 1 of the manuscript).

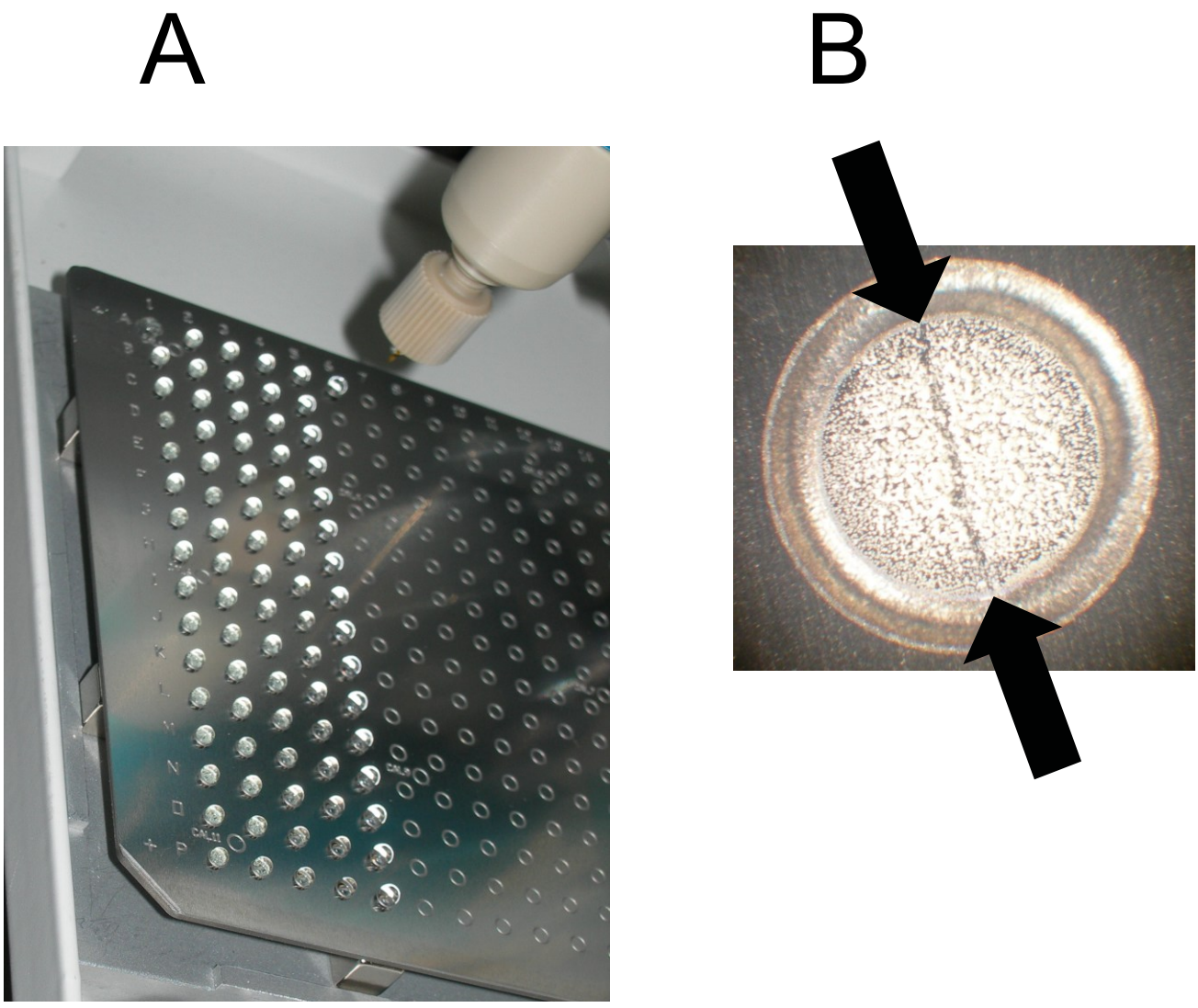


Figure S4 : A) Picture of an LC-MALDI deposition onto the hydrophobic plate, up to 4 µL can be sampled onto a 1700 µm target.

B) Picture of a MALDI spot after one acquisition in rastering mode, the groove generated by the laser appears between the two arrows.

Derivatization rate and ionization of SPITC derivatized peptides

Experimental

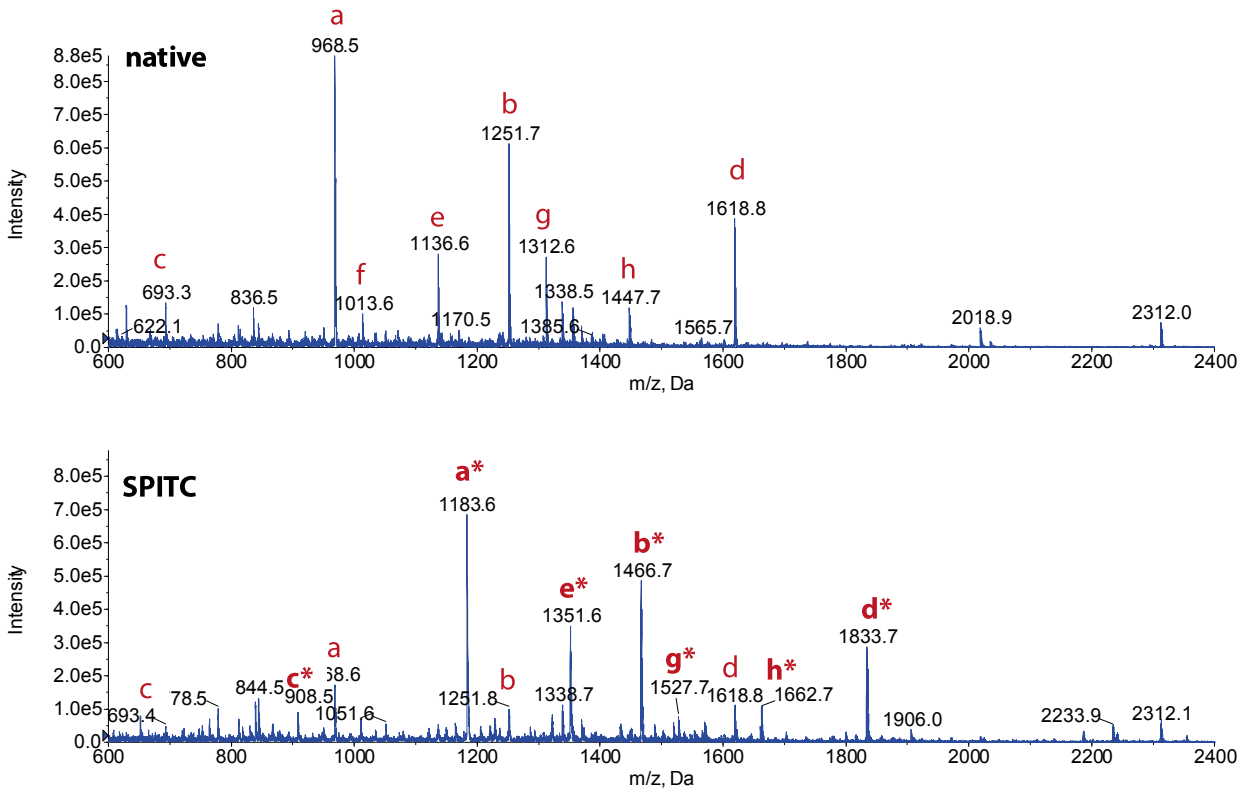
A solution of alcohol dehydrogenase (ADH1) from *S. cerevisiae* was prepared at 1 µg/µL in 50mM ammonium bicarbonate. An aliquot of the ADH1 solution (20 µL) was reduced, alkylated and digested by trypsin. Briefly, an amount of 10 µL of DL-dithiothreitol (50 mM in 50 mM ammonium bicarbonate) was added in the tube and incubated for 45 min at 60°C. Then, the reduction of cysteine was done by an addition of 10 µL of IAM (100 mM in 50 mM ammonium bicarbonate) and the incubation was performed in the dark at room temperature for 30 min. Before proteolysis, the sample was diluted by an addition of 60 µL of 50 mM ammonium bicarbonate and a proteomic grade trypsin was added to a final trypsin / proteins ratio of 1/40 (w/w). The digestion was performed overnight at 37°C and was stopped by the addition of 1 µL of neat formic acid.

A volume (10µL) of tryptic digest was kept native and another one (10µL) was submitted to 4-sulfophenylisothiocyanate derivatization. The volume was dried under vacuum, suspended in SPITC derivatization buffer (SPTIC 10 µg/µL, sodium bicarbonate 20 mM pH 10) and incubated for 45 min at 55°C. The derivatization was stopped by an addition of 5 µL of TFA aq. 1%. Both native and SPITC derivatized digests were desalted onto a C18 ZipTip, directly eluted in 20µL of CHCA (10 µg/µL, in 60/40 MeCN/TFA aq. 0.1%) and then spotted on the MALDI plate (1 µL by spot).

The MS full scans of the spot were acquired using the third quadrupole as a linear ion trap. The acquisitions were performed using the rastering mode.

Results

The SPITC-derivatized peptides are detected by a shift of 215 u from the *m/z* of their native forms. We cannot conclude about the derivatization yield because the peptides are chemically altered and the intensity changes relative to a peptide and its derivatized form do not necessarily correlate with their actual concentration changes. However, the SPITC derivatization preserves the overall signal to noise of the MS spectra and in most case the peaks of the native peptides were found to be 50-80% less intense in SPITC derivatized samples.



	native peptide	SPTIC peptide
a	EALDFFAR	**a***
b	SISIVGSYVGNR	**b***
c	EELFR	**c***
d	VLGIDGGEGKEELFR	**d***
e	GVIFYESHGK	**e***
f	ANELLINVK	
g	SIGGEVFIDFTK	**g***
h	VVGLSTLPEIYEK	**h***

Figure S5 MALDI-QqQ$_{LIT}$ MS full scan spectra of ADH1 tryptic digest under its native form and after SPITC derivatization. Q3 was operated as a LIT.

Supplemental references

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